University of Alberta

Characterization and Metal Dependence of *Helicobacter pylori* α 1,3/4 Fucosyltransferase

by

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ABSTRACT

Lewis antigens are well documented to be expressed on the lipopolysaccharide structures of *Helicobacter pylori.* While the function of these antigens is not well understood, they are of considerable interest, as they mimic the Lewis blood group antigens expressed by tissues of the human host. The *H. pylori* α 1,3/4 fucosyltransferase (FucT) is responsible for the final step of Le antigen synthesis. This thesis demonstrates the metal independence of FucT, and its tolerance to acidic pH. Mutation of the characteristic metal binding EXD motifs, well documented in many glycosyltransferases, did not abolish FucT activity. Although not metal dependent, moderate activation of FucT results with Mg^{+2} supplementation. The metal independent activity indicates that FucT is most likely a GT-B type glycosyltransferase.

Additionally, the cellular localization and the transcriptional patterns of FucT was assessed by immunofluorescent microscopy and RT-PCR. The microscopy probed for α1,3/4 FucT His_6 tag with fluorescently labeled antibody, allowing for a novel method of visualization of the cellular distribution of a bacterial FucT within the cell. As transcriptional analysis of the *fut* genes has not been previously assessed, RT-PCR was utilized to examine transcription patterns in Lewis antigen expressing and non-expressing strains.

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LIST OF ABBREVIATIONS

AIEX	anion exchange chromatography
ATP	adenosine triphosphate
ATCC	American Type Culture Collection
BabA	Lewis B binding adhesin
bp	base pair
BHI	brain heart infusion
CAZY	Carbohydrate-Active-enZYme
DD-Hep	D-*glycero*-D-*manno*-heptose
DNA	deoyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
FBS	fetal bovine serum
FucT	fucosyltransferase
GDP	guanosine diphosphate
GlcNAc	N-acetylglucosamine
GT	glycosyltransferase
GTP	guanosine triphosphate
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
ICP-MS	inductively coupled plasma mass spectrometry
IPTG	isopropylthiogalactoside
kb	kilobase
KDO	3-*deoxy*-D-*manno*-octulosonic acid
LacNAc	Galβ1,4-N-GlcNAc
LB	Luria-Bertani
Le	Lewis
Le^a	Lewis A
Le^b	Lewis B
Le^X	Lewis X
Le^Y	Lewis Y
LPS	lipopolysaccharide

MALDI-MS	Matrix-assisted laser desorption/ionization
MALT	Mucosal-associated lymphoid tissue lymphoma
mU	milliunit
MWCO	Molecular weight cut-off
NAP	neutrophil-activating protein
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PAI	pathogenicity island
PBS	phosphate buffered saline
PCR	polymerase chain reaction
rcf	relative centrifugal force
RNA	ribonucleic acid
SabA	sialylic acid binding adhesin
SD	Shine-Dalgarno
SDS	sodium dodecyl sulphate
UV	Ultraviolet
VacA	vacuolating cytotoxin
WT	wild type
YE	yeast extract

1 Introduction

1.1 *The Role of Helicobacter pylori in Disease*

Helicobacter pylori is a significant bacterial pathogen which inhabits the human stomach. Persistent colonization occurs in an estimated 50% of the global population, with a significantly higher prevalence observed in developing nations (Megraud *et al.*, 1989; Taylor and Parsonnet, 1995). Successful isolation and causal association of the bacterium with disease in humans was first demonstrated by Marshall and Warren in 1982 (Marshall and Warren, 1984). Initially, the bacterium was identified as belonging to the *Campylobacter* genus based on its curved, gram-negative rod appearance. The first classification of the bacterium was *Campylobacter pyloridis* (Marshall and Warren, 1984), however, following 16S rRNA sequence analysis, this classification was revised to place *H. pylori* in a new genus: *Helicobacter* (Romaniuk *et al.*, 1987).

Shortly after the initial identification of *H. pylori,* the bacterium was conclusively associated with incidents of gastric mucosal inflammation (Marshall *et al.*, 1985). It is well documented that *H. pylori* is a causative agent for a number of gastric ailments. The bacterium contributes to atrophic gastritis, peptic ulcer disease, and gastric cancer (Dunn *et al.*, 1997; Parsonnet *et al.*, 1991). Infection has also been linked to gastric non-Hodgkin's lymphomas (Parsonnet *et al.*, 1994) and gastric mucosa-associated lymphoid tissue (MALT) lymphoma (Eidt *et al.*, 1994). The correlation of *H. pylori* infection with gastric cancers is so strong that *H. pylori* has been designated a class 1 carcinogen by the World Health Organization, the only bacterial pathogen to date to receive this designation (IARC, 1994). Despite this ominous classification, it should be noted that most individuals colonized by *H. pylori* do not display symptoms of disease resulting from the infection. In addition, some areas of the world with very high infection rates also show low rates of gastric ulcers (Holcombe *et al.*, 1992; Megraud *et al.*, 1989). This illustrates that while *H. pylori* infection increases the risk of gastric ailments, it is not the single causative agent, and is not uniformly detrimental to the human host.

The pathology of *H. pylori* depends on a number of significant virulence determinants, including a Type IV secretion system encoded by the *cag* pathogenicity island (*cag*PAI) and secretion of the CagA protein, the flagella, prolific production of urease, and a variety of adhesins and antigenic structures (Prinz *et al.*, 2003).

1.2 *Epidemiology of H. pylori Infection*

Prevalence of *H. pylori* colonization and associated disease varies with geographical locale and socio-economic conditions. Rates of infection are lowest in children in the developed world, where less than 10% of youth currently become infected before adulthood (reviewed in Rothenbacher and Brenner, 2003). In contrast, in developing countries the percentage of the population colonized is drastically higher, and is suggested to be upwards of 70 - 90% (Rothenbacher and Brenner, 2003).

1.2.1 Prevalence of *H. pylori* in the Environment

There is very little evidence to date for an environmental reservoir of *H. pylori*. Studies, mainly from Japan, have given weak evidence to support the possibility of contaminated water sources carrying *H. pylori*. Some studies have shown high numbers of children testing positive for *H. pylori* infection near these waterways (Fujimura *et al.*, 2004; Horiuchi *et al.*, 2001; Sasaki *et al.*, 1999). The limitation of these studies being that they have been based only upon detection of *H. pylori*-specific DNA and not culture of *H. pylori*, and that river water from sources removed from human activity showed no evidence of *H. pylori* DNA (Fujimura *et al.*, 2004). One interpretation of these findings is that the presumptive *H. pylori* DNA detected may have originated from the surrounding human population, rather than from an environmental source. The prevalent view to date is that *H. pylori* resides only in its human host, and is neither found in the ambient environment nor carried by an intermediary species.

1.2.2 Modes of Transmission

H. pylori is found in all regions of the world, and thus is not spread from a point source in an epidemiological manner. Despite its prevalence in much of the global population, very little is understood about the precise mode of transmission. It has been hypothesized that the bacterium is transmitted via a fecal-oral route (Fox *et al.*, 1992; Thomas *et al.*, 1992), however many studies support an oral-oral mode of transfer (Allaker *et al.*, 2002);(Li *et al.*, 1996; Parsonnet *et al.*, 1999). In addition, there have been limited instances of iatrogenic transmission in institutional settings, most likely attributable to failures in endoscopic equipment sterilization (Langenberg *et al.*, 1990).

Despite a lack of understanding of the exact mechanism of transmission, trends of interfamilial transmission have been noted. Individuals are most likely to acquire *H. pylori* in the first five years of life, after which time infection rates drop dramatically (Rowland *et al.*, 2006). Children are at highest risk for infection when older siblings or the mother are infected, or when they are weaned from bottle feeding at a late age (Garg *et al.*, 2005; Rowland *et al.*, 2006). These patterns of transmission, coupled with the correlation of infection rates to lower socio-economic standards, would tend to support a fecal-oral or oral-oral route of transmission.

1.2.3 Geographic Variation

Regional differences are present in strains of *H. pylori,* as compared using DNA sequencing, which demonstrate the high degree of genomic diversity present in strains isolated from different geographic regions (Falush *et al.*, 2003). *H. pylori* strains have been classified into seven main populations that are reflective of these genomic sequence differences (Falush *et al.*, 2003). The genetic diversity of *H. pylori* is considered to be one of the highest of all known bacterial species (Cooke *et al.*, 2005; Taylor *et al.*, 1992). Sequencing of seven housekeeping genes and one virulence gene were used to study strain variation by Falush and coworkers (2003). Falush (2003) argued that these distinct *H. pylori* populations are reflective of human migration patterns and ethnic groups; however this view is not universally accepted. Regardless of how *H. pylori* followed the pattern of human migration and became endemic globally, it is interesting to note that on rare occasions, local populations may spontaneously clear the majority of *H. pylori* infection. Tokudome and coworkers (2005) documented small local populations in Indonesia with 1/50th of the prevalence of *H. pylori* infection compared to surrounding regions. The very low incidence of infection also corresponds to a very low rate of stomach cancer in these populations (Tokudome *et al.*, 2005).

1.3 *Bacteriology*

H. pylori is the only bacterium positively identified to successfully colonize the human stomach. Its survival in this extremely acidic environment is facilitated by a number of unique characteristics of the bacterium, including the production of urease (Dunn *et al.*, 1990; Sjostrom and Larsson, 1996) and the ability to encapsulate in an impenetrable casing referred to as the coccoid form (Chan *et al.*, 1994). *H. pylori* survives poorly in acidic conditions, instead preferring the more neutral environment of the mucosal layer lining the stomach, where the pH is thought to vary between pH 4.0 and 6.5 (Chu, 1999). This mucosal layer which shields the gastric epithelium from the ravages of acidic stomach secretions also provides for a neutral environmental niche for the bacterium to inhabit. Thus, crucial to successful colonization are *H. pylori*'s flagella, which facilitate the movement of bacteria through the gastric mucosa (Ottemann and Lowenthal, 2002), and protection by the secretion of urease, which is thought to neutralize the immediate surroundings of the bacterium (Sjostrom and Larsson, 1996).

The metabolic requirements of *H. pylori* are not fully elucidated. The bacteria appear not to require carbohydrates as an energy source (Schilling *et al.*, 2002). Using a bioinformatics approach, it was suggested that the minimum medium requirements consist of 8 amino acids (Schilling *et al.*, 2002). As such, a defined media for culture of *H. pylori* is not available. Typically, a rich medium, supplemented with whole blood or serum is required for growth. The function of this supplementation may be for nutritional purposes, however the serum may also function to detoxify the media (Dunn, 1997).

Iron uptake is a significant environmental stimulus to *H. pylori* in the gastric environment (van Amsterdam *et al.*, 2004; Husson *et al.*, 1993; Worst *et al.*, 1995). While essential for survival, as the co-factor of many enzymes and a catalyst for the electron transport process, iron can also be very toxic to the organism in the presence of oxygen. The result of reaction of iron with O_2 is the formation of toxic free radicals (Wang *et al.*, 2005). As such, the ability of *H. pylori* to efficiently take up excess iron from its environmental niche appears to be vital to its survival (van Amsterdam *et al.*, 2004).

1.3.1 Urease Production

The survival of the bacterium at very acidic conditions (≤ pH 4.0) is dependent on the production of urease (Sjostrom, and Larsson, 1996). This apoenzyme is expressed constitutively at very high levels, accounting for as much as 15% of the total protein synthesized by the bacterium (Mobley, 1995). In addition to facilitating survival, it has been suggested that urease may function directly as a virulence factor. It has been shown to disrupt the gastric epithelial cells and to possibly also induce an immune response (Harris *et al.*, 1996).

1.3.2 Flagella

A second factor required for *H. pylori* persistence in the stomach are the flagella which allow for motility. To facilitate the bacterium's passage to this unique environmental niche, the bacteria contain multiple flagella which propel the bacteria through the thick mucous layer. A set of 4-6 sheathed flagella are found at one pole of the bacterium and are anchored to the membrane with a terminal bulb extension of the sheath. The flagella measure 30 μM in length and approximately 2.5 nm in width. The presence of functional flagella is essential to successful colonization, as mutant non-motile strains are unable to colonize the stomach. (Eaton *et al.*, 1992; Eaton *et al.*, 1996; Ottemann and Lowenthal, 2002)

1.3.3 Coccoid Form

Under certain adverse growth conditions, or during prolonged *in vitro* culture, *H. pylori* cells will change into a coccoid form (Bode *et al.,* 1993; Catrenich and Makin, 1991). There has been considerable controversy over whether or not these cells are viable (Eaton *et al.,* 1995; Hua, and Ho, 1996; Kusters *et al.,*1997; Ren, 1999). Some evidence suggests they are metabolically active and less amenable to culture (Cellini *et al.,* 1994; Hua, and Ho, 1996; Ren, 1999). The coccoid form is dormant and less able to infect a potential host than the spiral rod *H. pylori* (Eaton *et al.,* 1995). Recently, gene expression studies of coccoid *H. pylori* cultures have claimed differential virulence gene expression in coccoid versus spiral bacterial cultures (Monstein, 2001; Nilsson *et al.*, 2002). One potential problem with the studies demonstrating the viability of the coccoid bacteria is the challenge in determining whether or not a bacterial culture consists of uniformly coccoid cells. Nilsson *et al.* (2002) documents the presence of three distinctive morphologies in culture after 16 days of culture, but considered only one of these as being representative of a degenerative coccoid form. Thus, the subsequent conclusion that the coccoid form represents a viable bacterium must take into consideration that the indications of viability observed may result from a small population of spiral bacteria which do not possess coccoid morphology.

1.3.4 Cell Membrane & Lipopolysaccharide

H. pylori expresses large amounts of outer membrane proteins (OMPs). Urease and HspB are both abundant in preparations of the outer membrane layer. Additional OMPs expressed include a family of porin proteins and iron-uptake proteins (reviewed in Dunn, 1997).

The lipopolysaccharide (LPS) of *H. pylori* has the typical structure found in Gram negative bacteria (Moran *et al.,* 1992; Moran, 1996). One feature of particular relevance to these studies is the expression of Lewis (Le) antigens on the LPS (Aspinall *et al.*, 1994; Moran, 1996) (see section 1.8). LPS and in particular the O-antigen structure is necessary for induction of host immune responses (Eaton *et al.*, 2004). *H. pylori* strains lacking O-antigen have been shown repeatedly in animal models to be impaired in their to colonize gastric epithelial tissue (Altman *et al.*, 2003; Eaton *et al.*, 2004).

1.4 *Genome Characteristics*

The genomes of two *H. pylori* strains, 26695 and J99, have been completely sequenced (Alm *et al.*, 1999; Tomb *et al.*, 1997). In addition, genome sizing using restriction mapping combined with pulsed-field gel electrophoresis of genomic DNA and cosmid library studies has been completed for a number of strains (Bukanov and Berg, 1994; Jiang *et al.,* 1996; Taylor, *et al.* 1992). The *H. pylori* genome size is relatively small, ranging from 1.6 to 1.73 Mb, and there is a fairly high level of plasticity between both the size and gene content of the strains analyzed to date (Alm *et al.* 1999). Analysis of the two complete genome sequences has shown a significant number of strain specific genes, comprising approximately 6-7% of the total genome (Alm *et al*, 1999).

Several notable features of the *H. pylori* genome have been identified (Alm *et al.*, 1999; Ge and Taylor, 1999; Wang *et al.*, 1999b). The first is the high proportion of the genome (~ 1%) which encodes OMPs, potentially involved in host-bacteria interactions (Alm, *et al.* 1999; Ge and Taylor, 1999; Tomb *et al.*, 1997). In addition, the genome contains several homologs of common bacterial DNA restriction/modification systems; as well as a short region of the genome comprising a plasticity zone which contains almost 50% of the strain specific sequences. Finally, and of particular note, is the presence of homopolymeric tracts and dinucleotide repeats which are commonly observed in *H. pylori* genes encoding cell surface structures, LPS synthesis enzymes, and DNA restriction/modification system components (Ge and Taylor, 1999; Wang *et al.*, 1999b).

The phenomenon of phase variation in gene translation is prevalent in *H. pylori* due to these polymeric tracts and dinucleotide repeats. For example, the presence of poly-C tracts in the 5' end of the gene coding region results in different LPS antigenic profiles for a single strain of *H. pylori* (Appelmelk *et al.*, 1998). Phase variation of LPS structures has been documented in other bacteria, namely *Neisseria* spp. and *Haemophilus influenzae* (Apicella *et al.*,1987; Weiser *et al.*,1990). Antigenic variation is a result of the polymeric tracts leading to slip strand synthesis during DNA replication, which in turn acts to switch gene expression on or off (Wang *et al.*, 2000). Thus, the insertion or deletion of an adenine or cytosine residue during DNA replication leads to a shift in the open reading frame (ORF), which can result in a truncated gene sequence.

The high level of phase variation in *H. pylori* allows for antigenic variation without deletion or insertion of new ORFs, and thus may reduce selective pressure on the bacterium to develop further strain specific genes. (See 1.7.4; For a complete review of *H. pylori* genome characteristics, please refer to Ge and Taylor, 1999.)

1.5 *Virulence Factors*

Virulence factors may be defined as pathogen characteristics which facilitate its ability to induce disease. *H. pylori* has a variety of adaptations which aid its survival in the stomach and contribute to its virulence. The most often cited virulence determinants of *H. pylori* are the vacuolating cytotoxin A (VacA) and cytotoxin-associated gene A (CagA) proteins (Blaser and Atherton, 2004; Cover and Blaser, 1992; Crabtree *et al.*, 1991). CagA is encoded as part of a Type IV secretion system, contained on the *cag*PAI (Covacci, *et al.*, 1999). The *cag*PAI is suspected to also encode other effector molecules that contribute to the virulence of these strains (Odenbreit, 2000). A number of other adhesion and secreted factors have also been investigated for their contribution to the pathogenicity of *H. pylori*. These additional factors include the flagella, BabA adhesin, and Le antigens (Blaser and Atherton, 2004).

As the two major virulence factors characterized to date, CagA and VacA expressing strains of *H. pylori* isolates are defined as Type I strains. By this definition, Type II strains which express neither marker are generally considered to be less virulent (Covacci *et al.*, 1993; Ghiara *et al.*, 1995).

1.5.1 Vacuolating Cytotoxin

VacA is a virulence factor found to be widely expressed in nearly half of all *H. pylori* strains (Cover and Blaser, 1992). The 88 kDa VacA protein may be secreted as soluble protein, or may remain localized to the surface of the bacterial outer membrane (Cover and Blaser, 1992). When secreted into the extracellular space, the soluble VacA forms oligomeric structures (reviewed in Cover and Blanke, 2005). Secreted VacA in its oligomeric form can insert into planar lipid bilayers of the host cell to form anion-selective channels. VacA is capable of inducing a number of changes in host epithelial cells, including vacuolation and cell death (Megraud, 2001). In addition, VacA is found to influence immune cell activity, particularly T lymphocytes. This may be attributable to the disruption of membrane integrity by breaching the host cell membrane and disrupting calcium ion influx (Gebert *et al.*, 2004). The result of VacA vacuolization of T cells is immunosuppressive in nature, as it down regulates the production of interleukin 2, which is required for T cell proliferation (Boncristiano *et al.*, 2003; Gebert *et al.*, 2004).

While the *vacA* gene sequence is present in the vast majority of all *H. pylori* strains, the gene is not necessarily expressed. Production of VacA depends upon the signal sequence present (*s1a, s1b, s1c, s2*) and mid-region (*m1, m2*) of the *vacA* gene (Megraud, 2001). Strains of *H. pylori* isolated from different human populations exhibit differing prevalence of the *vacA* alleles (Achtman *et al.*, 1999; Van Doorn *et al.*, 1999). The signal sequence, located at the N-terminus of the protein is of particular importance in determining the vacuolating cytotoxicity of the strain. VacA molecules with the *s1* sequence exhibit a high level of potency, whereas strains with the *s2* sequence do not exhibit any vacuolating cytotoxicity. The mid region (*m1* or *m2*) also appears to play a role, with the most potent VacA consisting of the s1/m1 form (Letley and Atherton, 2000; McClain *et al.*, 2001). As might be expected, individuals colonized by *H. pylori* with the *vacA s1* allele are the most likely to develop peptic ulcer disease and gastric cancer (Atherton *et al.*, 1995; Figueiredo *et al.*, 2001; van Doorn *et al.*, 1998; van Doorn *et al.*, 1999).

1.5.2 Neutrophil-activating Protein

Infection with *H. pylori* is well documented to cause chronic inflammation in the host, which may or may not become symptomatic over time. This inflammation is associated with the infiltration of phagocytes (particularly neutrophils) to the gastric mucosa (Craig *et al.*, 1992). The *H. pylori*-neutrophil activating protein (HP-NAP) has been shown to promote the infiltration of neutrophil infiltration and adhesion to gastric endothelial cells (Evans *et al.*, 1995). HP-NAP is a large iron-binding protein which interacts through glycoconjugate structures on the membrane of human cells (Teneberg *et al.* 1997; Tonello *et al.* 1999).

1.5.3 *cag* Pathogenicity Island

The *cag*PAI is a well documented virulence factor, associated with the development of peptic ulcer and gastric cancer (Blaser *et al.*, 1995; Nomura *et al.*, 2002). Encoded on the GC-rich 40 kb insertion in the *H. pylori* genome, the *cag* PAI encodes a type IV secretion system capable of injecting effector molecules into the host cell (Odenbreit *et al.*, 2000). There are more than 30 genes encoded on the *cag*PAI, several of which have been characterized or show homologies to type IV secretion system proteins (Covacci *et al.*, 1999; Tummuru *et al.*, 1995). CagA is the only documented effector molecule to date; however, it is probable that other unidentified effectors exist, as other type IV systems have more than one effector. CagA is a 128 kDa protein (Crabtree *et al.*, 1991), often present in *H. pylori* strains isolated from individuals suffering more severe gastric diseases (ie. gastric ulcers and cancers) (Covacci *et al.*, 1993). CagA is injected by the secretion system into the host cell, where the protein is then tyrosine phosphorylated. The phosphorylated form of CagA triggers as series of biochemical and morphological changes in the host cell, leading to the "hummingbird" morphology (Segal *et al.*. 1999). Recently, a second protein from the *cag*PAI has been shown to be required for translocation of CagA, however it does not appear to cross into the host cell (Couturier *et al.*,2006).

1.5.4 Adherence Factors

There has been considerable debate and some difficulty in determining the factors used by *H. pylori* to adhere to the gastric epithelium. Several factors involved in adherence have been extensively characterized, including the blood group binding adhesion protein (BabA) (Ilver *et al.*, 1998), sialic acid-binding adhesin (SabA) (Mahdavi *et al.*, 2002) and the Le antigens, which may be present on the surface of both *H. pylori* and the cells of the host gastric epithelium (Appelmelk *et al.*, 1996; Ilver *et al.*, 1998; Mahdavi *et al.*, 2002).

1.5.4.1 BabA Adhesin

BabA was the first well characterized *H. pylori* protein shown to facilitate adhesion to the human stomach. BabA has been demonstrated to facilitate binding of *H.*

pylori to gastric epithelial tissue by adherence to host expressed Lewis B (Le^b) antigen. BabA is a 75 kDa protein which localizes to the bacterial outer membrane (Ilver *et al*, 1998). Interestingly, BabA is very specific to H-type 1 (H-1) and Le^b antigens. The original discovery and characterization of BabA found that none of the 95 isolates examined were able to bind to related Lewis antigens (Ilver *et al.*, 1998). Two *babA* genes have been identified, only one of which, denoted *babA2*, results in a functional BabA protein. Evidence has been presented that *babA2* and *cagA* strains are associated with gastric ulcer, atrophic gastritis and gastric carcinoma (Gerhard *et al.*, 1999; Oliveira *et al.*, 2003; Prinz *et al.*, 2001). This is indicative of the possible role of Le antigen expression by the host in the persistence of *H. pylori* infection.

1.5.4.2 SabA Adhesion

The sialic acid binding protein SabA, is found to bind sialylated Le^X antigens expressed during chronic inflammation of the gut (Mahdavi *et al.*, 2002). SabA is an outer membrane protein, which binds to carbohydrate in a similar manner to BabA. It has, however, been demonstrated to be expressed independently of BabA, and seems to also have a weaker adhesion to gastric tissue. The expression of SabA is interesting, considering the high levels of sialylated glycoconjugates that are present in diseased gastric tissue, including gastric cancer (Amado *et al.*, 1998).

1.5.4.3 Lewis Antigens

Identical Le antigens are expressed by both the bacterium and the host, in an uncommon case of molecular mimicry between bacterium and host. Several studies have investigated the relationship between Le antigen expression by the bacterium and the incidence of pathological symptoms in the host (Heneghan *et al.*, 2000; Lozniewski *et al.*, 2003; Wirth *et al.*, 1996). To date, the studies have indicated the bacterial Le antigens may facilitate adhesion at the later stages of attachment, and may be correlated with presentation of pathogenic symptoms (Edwards *et al.*, 2000; Heneghan *et al.*, 2000; Wirth *et al.*, 1996). However, Le antigens are not an absolute requirement for colonization or virulence (Rasko *et al.*, 2000). As such, there is not complete consensus as to whether or not the Le antigens constitute a true virulence factor (Mahdavi *et al.*, 2003)

1.6 *Biological Effects of H. pylori*

1.6.1 Effect on Host

The harmful outcome of *H. pylori* colonization on the host is due to a number of influences that *H. pylori* exerts on the gastric epithelium. Infection leads to chronic gastritis and to epithelial-cell proliferation, by causing reduced expression of certain cell-cycle regulation proteins, and by inducing certain host response mechanisms including increased secretion of gastrin, a mucosal secreted hormone, and stimulation of cell proliferation in the gut (reviewed in Peek and Blaser, 2002; Fan *et al.*, 1995; Lynch *et al.* 1995). Gastritis results from an inflammatory host response, which involves activation and secretion of a number of pro-inflammatory cytokines including TNF-α, interferon-γ, and IL-I (Holck *et al.*, 2003; Moss *et al.*, 1994). A third harmful effect of infection is the disruption of normal apoptosis in the gastric epithelium. *In vitro* assays have shown repeatedly that *H. pylori* stimulates apoptosis of gastric cells (Peek *et al.* 1999). This effect is variable, depending on both the human host population and bacterial strains (Peek *et al.* 1997). In some instances, the chronic gastritis caused by infection eventually progresses towards more serious pathologies including peptic ulcers, atrophic gastritis and gastric cancer in a subsection of *H. pylori* colonized individuals.

1.6.2 Diagnosis & Treatment of *H. pylori* Infection

When patients present with symptoms of gastric distress they may be tested for *H. pylori* colonization and treated with a series of antibiotics if infection is found (NIH consensus panel, 1994). Testing methods may be subdivided into invasive and non-invasive tests. Invasive tests, i.e. endoscopy, followed by a rapid urease test upon which treatment is based is the most common method of diagnosis. Usually a histological examination of biopsy tissue is also performed, and, more rarely, culture of bacteria and PCR probing for *H. pylori* specific genes (Genta *et al.*, 1994; Hachem *et al.*, 1995; Marshall *et al.*, 1987; el-Zaatari *et al.*, 1995). Non-invasive methods have also been reported to give high levels of sensitivity, and include the ^{13}C urease breath test, serotyping, PCR probing of saliva and detection of *H. pylori* specific antigen in stools (Monteiro *et al.*, 2001; Li, *et al.*, 1996 Klein *et al.*, 1996; Hirschl *et al.*, 1996; Marchildon *et al.*, 1996; Peura *et al.*, 1996; Stubbs and Marshall, 1993; Perez-Perez, 1988). The advantages of the non-invasive methods include decreased cost, immediate results in some instances, and reduced risk to the patient. The advantage of bacterial culture is that it allows antibiotic susceptibility of the infecting strain to be determined.

Patients who are infected with *H. pylori* undergo a rigorous regime of treatment with a series of antibiotics and proton pump inhibitors. Typically two antibiotics (clarithromycin and either amoxicillin or metronidazole) will be administered in addition to a proton pump inhibitor (Vakil, 2006). Bismuth citrate has also been found to be an effective agent, in combination with proton pump inhibitors and appropriate antibiotics (Van Caekenberghe and Breyssens, 1987).

There has been increasing concern about antibiotic resistance in *H. pylori*, particularly to two of the antibiotics commonly used to treat infections: metronidazole, and clarithromycin. Resistance rates in North America are increasing, and are especially high for metronidazole ranging from 4 -31% in various geographical regions of the United States (Duck *et al.*, 2004). In addition, Duck *et al.* (2004) found that 12.9% of the strains were also resistant to clarithromycin. Amoxicillin resistance is still uncommon (Duck *et al.*, 2004). While clearly an issue in North America, very high rates of resistance are also observed in developing countries. Extremely high levels of metronidazole resistance have been observed in India, and have also been documented in Africa and regions of South America (Alarcon, *et al.*, 1999; Mukhopadhyay, *et al.*, 2000). Given these extraordinarily high rates of resistance, there is significant interest in developing novel antibiotics and therapies for combating *H. pylori* infection.

1.7 *Lipopolysaccharide*

1.7.1 Structure of Lipopolysaccharide

Bacterial LPS synthesis is vital to the maintenance of proper cell surface integrity and recognition, both between members of the bacterial community, and by the host organism (Lerouge and Vanderleyden, 2001). The *H. pylori* LPS structure is typical of a Gram negative bacterium (Fig 1.1) and is based on the following three components: lipid A, core oligosaccharide, and O-antigen. The lipid A component anchors the oligosaccharide to the outer cell membrane and consists of disaccharide moieties linked to the terminal phosphate groups of the fatty acid chains in the membrane. The core

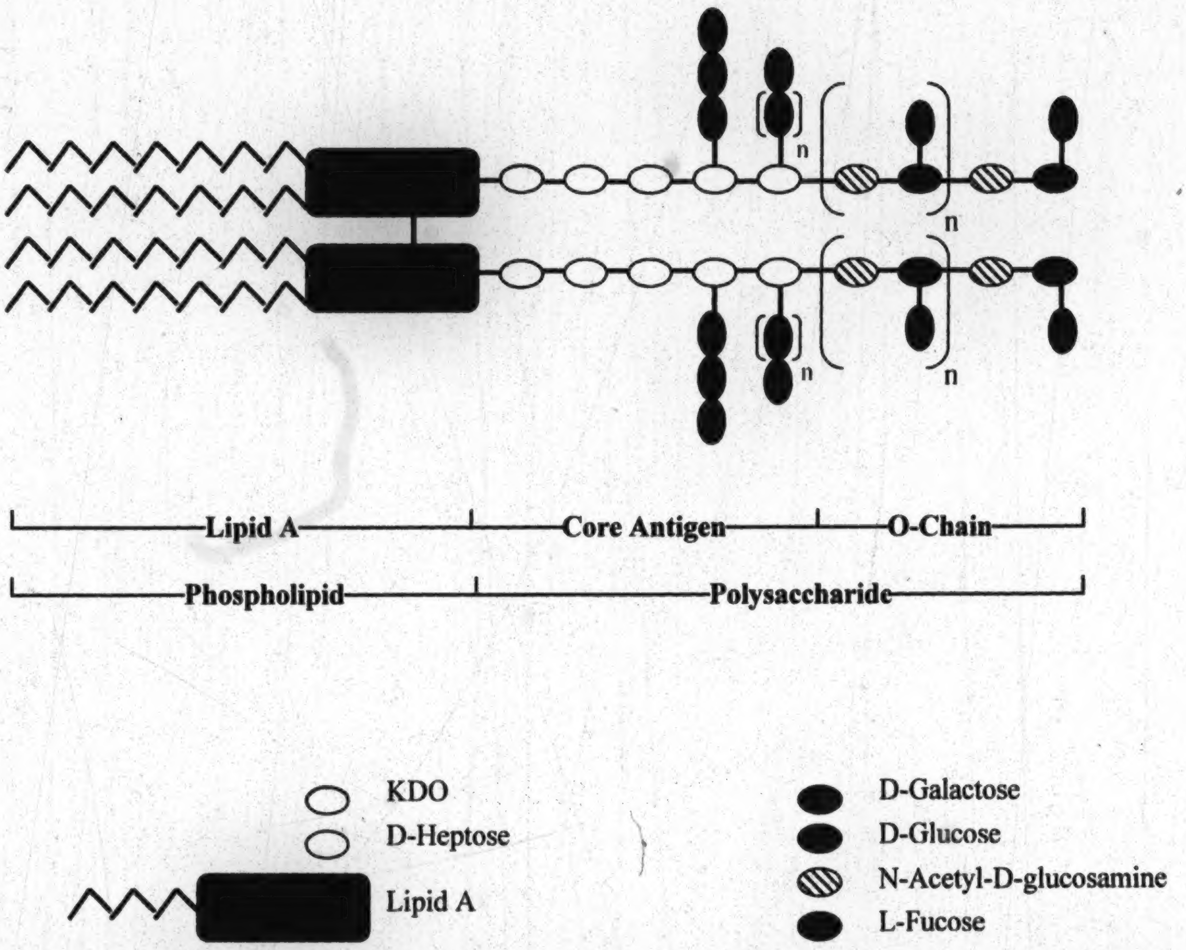

n
n
n
n
Lipid A
Core Antigen
O-Chain
Phospholipid
Polysaccharide
KDO
D-Heptose
Lipid A
D-Galactose
D-Glucose
N-Acetyl-D-glucosamine
L-Fucose

Figure 1.1 Structure of lipopolysaccharide. The three main portions of the lipopolysaccharide molecule are the lipid A, the core antigen saccharide and the terminal O-antigen portion. The core antigen is quite conserved within a species, however the O-antigen may be highly variable. KDO – 3-deoxy-D-*manno*-2-octulosonic acid.

oligosaccharide is a relatively conserved structure consisting of sugars and sugar derivatives. The O-antigen polysaccharide is a variable antigenic structure that branches off the core oligosaccharide, extending from the cell surface. It is made of repeating oligosaccharide units, typically 3-6 sugars in length (O-units). There will often be 10 – 30 O-units making up the O-antigen molecule (reviewed in Reeves *et al.*, 1996). In *H. pylori*, the terminal end of the O-antigen typically carries a specific fucosylated structure; one or more of four Le antigens. This is in contrast to other well characterized Gram negative bacteria such as *E. coli* and *Salmonella*, which tend to have highly polymorphic O-antigen structures (Reeves *et al.*, 1996).

1.7.2 Glycotypes of *H. pylori*

Monteiro *et al.* (2000) devised a classification for *H. pylori* glycoforms that consists of 8 glycotype families (Table 1.1). These families were determined by comparing the LPS chemical structures of a number of *H. pylori* strains, and grouping them by conserved structures. The most common side chains are fucosylated, however glucosylated and galactosylated LacNAc chains are characteristic of glycotypes D and E. While these glycoforms represent the immunogenic profile of an *H. pylori* strain at any given time point, it has been documented that the glycotype of an *H. pylori* strain can vary over time and may be influenced by environmental stimuli such as growth medium and pH (Monteiro *et al.*, 2000; Moran, 1995; Moran *et al.*, 2002).

Table 1.1 Classification of *H. pylori* lipopolysaccharides into glycotype families. Data shown is adapted from Monteiro *et al.* (2000). Note that some strains of *H. pylori* have been observed to express different glycotype families when cultured in the laboratory. Strains in **bold** have been used in the studies comprising this thesis.

Glycotype	Antigen blood group type	Terminal Le antigen	O-antigen	DD-Heptan core	Strain	LPS Form
A	Type 2	Le^{X}	Le^{X}	core	NCTC11637	Smooth
		Le$^{X/Y}$	Le^{X}	core	P466	
		Sialyl Le^{X}	Le^{X}	core		
		Le^{X}	Le^{X}	core	**26695**	
		Le$^{X/Y}$	Le^{X}	core	**J99**	
B	Type 2	Le$^{X/Y}$	Le^{X}	DD-Heptan core	O:3	Smooth
C	Type 2	Le^{Y}	no internal	DD-Heptan core	O:6	Smooth
D	Type 2	α-Glc	LacNAc	core	UA861[*]	Smooth
E	Type 2	α-Glu	LacNAc	core	471[†]	Smooth
F	Type 1 & 2	Le$^{a/X}$	Le^{X}	core	**UA948**	Smooth
		Le$^{dis/X/Y}$	Le^{X}	core	UA955	
G	Type 1 & 2	Le$^{dis/X/Y}$	no internal	core	**26695**	Semi-rough
		Le^{Y}		core	SS1	
H	Type 1	Le$^{b/d}$	no internal	core	UA913	Semi-rough

[*] UA861 expresses one internal Le^{X}. [†] Strain 471 contains fucosylated GlcNAc units. Le^{dis} – Lewis disaccharide

1.7.3 Gene Organization & O-antigen Clusters

LPS synthesis involves a series of glycosyltransferases and sugar-nucleotide synthesis reactions. Typically in Gram negative genomes, the genes coding for the proteins required for synthesis of the LPS precursors and transferase reactions required to assemble to final antigenic structures are clustered, co-regulated and often co-transcribed (Reeves *et al.,* 1996). The common clusters can be classified into three main categories of genes: those for biosynthesis of the nucleotide sugars required for polysaccharide synthesis; those for glycosyltransferase processes; and processing genes. Typically, the genes involved with the synthesis of the core oligosaccharide, as well as the antigenic side chains (ie. O-antigen) tend to be co-regulated and co-transcribed (Samuel and Reeves, 2003). Some well studied examples of this include the *E. coli rfa* (LPS core) and *rfb* (O-antigen), and the *Salmonella* sp. *rfb* (O-antigen) clusters, all of which contain genes involved in the synthesis of a finite portion of the LPS structure (reviewed in Samuel and Reeves, 2003).

While the genes for synthesis are clustered, the LPS produced by different strains of a species tend to have an extremely high degree of antigenic diversity. For example, there have been at least 186 O-antigen serotypes documented for *E. coli* (Samuel and Reeves, 2003). A survey of the annotated *H. pylori* genomes in the TIGR comprehensive database reveals that the genes for LPS synthesis are scattered throughout the genome. There are small clusters of O-antigen synthesis related genes. *H. pylori wbcJ*, *algA*, and *rfbD* are grouped together, apparently in the same operon, and all appear to be involved in the conversion of GDP-D-mannose to GDP-L-fucose (the nucleotide sugar donor in Le antigen synthesis) (McGowan *et al.*, 1998). However, these genes form a much smaller cluster than those observed in other enteric Gram negative bacteria, which encode an O-antigen (*rfb*) gene cluster from 11 to 18 genes in length (Samuel and Reeves, 2003).

1.7.4 Phase Variation of *H. pylori* LPS

Phase variation refers to mechanisms used by several mucosal pathogens including *Neisseria* spp. and *H. pylori* to vary the expression of cell surface structures by the reversible ON/OFF switching of gene expression (Ge and Taylor, 1999). The Le antigens undergo frequent phase variation, at a rate of 0.2-0.5%, altering the Le phenotype of colonies derived from a single isolate (Appelmelk *et al.*, 1998). Rasko *et al.* (2000) showed that frequent phase variation of Le antigen expression in *H. pylori* may occur over the course of colonization in some individuals.

It has been suggested that the fucosyltransferase (FucT) protein expression level of the bacterial strain directly influences the Le antigen expression pattern. Expression of the FucT can vary by typical phase variation mechanisms, including slipped-strand synthesis and di-nucleotide repeats. The presence of poly-A and poly-C tracts can lead to slipped-strand mispairing during DNA replication which results in frameshift mutations, leading to silencing of gene transcription (Appelmelk *et al.*, 1999; Ge *et al,* 1999). The reversible ON-OFF switching for the *fut* genes occurs with high incidence due to the presence of poly(C) tracts in the DNA which may create stop codons, leading to a consequently shortened ORF. These regions are termed "hypermutatable" by Wang *et al.* (2000).

A second mechanism employed by the bacterium to alter the LPS antigen expression is (-1) ribosomal frameshift which has been documented for the α1,2 fucosyltransferase of *H. pylori* (Wang *et al.*, 1999b). In the mid-region of the gene, an internal Shine-Dalgarno (SD) sequence and a poly-A containing heptamer (AAAAAAG), is followed by a potential stem-loop structure (Wang *et al.*, 1999b). The heptamer is very susceptible to -1 reading frame slippage during ribosomal translation. The probability of this occurring is further increased by the presence of the SD sequence and potential stem-loop structure, as they may also interact with the ribosome. The net result is that the gene is expressed with a high degree of variability, thereby leading to an altered LPS antigenic profile.

1.7.5 Influence of Environmental Stimuli on LPS synthesis

The expression of LPS has been shown to be affected by environmental stimuli, including pH, and culture conditions (McGowan *et al.*, 1998). There is also extensive local variation of Le antigen expression by *H. pylori* in different regions of the stomach (Nilsson *et al.*, 2002; Rasko *et al.*, 2000b).

The expression patterns of LPS of some bacterial species have been shown to be indirectly influenced by Mg^{2+} levels. The two component PhoP/PhoQ signaling cascades of *Salmonella typhimurium* or *E. coli* are two examples of systems which lead to different gene expression levels in the presence of altered extracellular Mg^{2+} levels (Gibbons *et al.*, 2005; Winfield and Groisman, 2004). Whether Mg^{+2} is significant in the regulation of expression of the FucT genes of *H. pylori* is not known.

1.8 *Lewis Antigens*

Le antigens are fucosylated antigenic structures expressed on the surface of both human gastric epithelium and *H. pylori* (Taylor *et al.*, 1998). It has yet to be fully elucidated exactly what role the bacterial Le antigens play in the course of human colonization. Taylor *et al.* (1998) found there was no direct correlation between the Le antigens expressed by *H. pylori* and the human host. However, given that more than 80% of *H. pylori* strains typically express Le antigens (Simmons-Smit *et al.*, 1996), it seems probable that Le expression plays an important role in facilitating colonization of the stomach. The findings of Taylor *et al.* (1998) are in contrast to those of Writh *et al.* (1996), who found expression of *H. pylori* Le antigen expression was associated with the *cagA* and *vacA* genotype, and that these strains were more common in individuals suffering from ulcer disease than in asymptomatic colonized individuals. The high percentage of strains expressing Le antigens is particularly significant when one considers that other Gram negative bacteria (eg. *E. coli* and *Salmonella enterica*) do not have highly conserved O-antigen structures (Lerouge and Vanderleyden, 2001). There are several lines of evidence pointing to a role for Le antigens in *H. pylori* pathogenesis, however their exact role has yet to be determined. Three main roles have been suggested as the function of *H. pylori* Le antigens: (i) induction of autoimmune response to facilitate colonization, (Appelmelk, *et al.,* 2000; Appelmelk, *et al.,* 1996) (ii) evasion of immune response (Moran, *et al.*, 2002), or (iii) an adhesive function (Appelmelk, 2000; Edwards *et al.,* 2000; Lozniewski, *et al.,* 2003). The first two options both involve a molecular mimetic role of the bacterial antigens to those expressed by the host.

1.8.1 Molecular Mimicry

The first two significant suggestions as to why *H. pylori* expresses identical Le antigens to the host are associated with the concept of molecular mimicry. The first proposal is that bacterial Le antigens may facilitate the induction of an autoimmune response in the host, however, the potential autoimmunity mediated by the bacteria has not been experimentally confirmed (Faller, 1998; Kamiya, 1999). Proponents of this school of thought stress that *H. pylori*, relative to other bacterial pathogens, expresses mostly smooth LPS, which is less immunogenic than rough LPS (Monteiro, 2000; Moran *et al.*, 1996). The second suggestion is that mimicry of host antigens by the bacterium allows the bacteria to be more capable of actually evading the host immune response, and that the Le antigens therefore facilitate colonization by *avoiding* induction of an immune response. The true scenario may lie somewhere between these two suggestions. There has been a long history of co-existence between the host and bacterium. It is probable that both the host and bacteria have exerted selective pressure on each other. Taken together, the evidence supporting these suggestions strongly suggests that the expression of these conserved immunogenic structures, mimicking those of the host, play an important role in the pathogenesis of *H. pylori* (Mahdavi *et al.*, 2003).

1.8.2 Adherence to Gastric Epithelium

H. pylori Le antigen expression may act to facilitate binding of the bacterium to gastric epithelial cells. Studies by Edwards *et al.* (2000) have shown the adherent properties of Le^X to gastric epithelium. In addition, *H. pylori* strains lacking O-antigen Le structures have been shown experimentally to have an impaired ability to colonize in animal models of infection (Martin *et al.*, 2000). Taylor *et al.* (1998) obtained immunoelectron micrographs using Le^X antibody which showed *H. pylori* expressing Le^X structures attaching to gastric epithelial cells. Taken together, these studies indicate that the Le antigens are involved in bacterial adherence to gastric epithelium.

1.8.3 Lewis Antigen Biosynthesis

Le antigens in *H. pylori* are expressed on the sub-terminal and terminal ends of the O-antigen side chains of the LPS. A transfer of fucose to the O-antigen backbone is the final stage in synthesis of the Le antigens; a reaction catalyzed by the bacterial FucT. Transfer of GDP-fucose is also the final stage in the mammalian synthetic pathway of Le antigens. The respective synthetic pathways of *H. pylori* and human Le antigen synthesis are shown in Fig. 1.2.

1.8.4 Fucosyltransferase & *fut* Gene Characteristics

Analysis of the genome sequences from *H. pylori* strains 26695 and J99 has shown the presence of three FucT genes; two α1,3/4 FucT (encoded by *futA* and *futB*) and an α1,2 FucT (encoded by *futC*) (Ge and Taylor, 1999; Tomb *et al.*, 1997). *futC,* encoded on the 26695 genome is predicted to be truncated, however low expression of intact α1,2 FucT still occurs due to the phenomenon of translational frameshifting (Wang *et al.*, 1999). The three FucT genes show very low homology to mammalian FucTs (Ge *et al.*, 1997; Wang *et al.,*1999). There is a higher level of conservation between α1,2 FucT sequences than between α1,3/4 FucTs; both between different *H. pylori* strains, and with mammalian counterparts (Ge *et al.*, 1997; Wang *et al.,*1999). The *H. pylori* FucT genes have been cloned, overexpressed, and well characterized by our laboratory (Chan *et al.*, 1995; Ge *et al.*, 1997; Ma *et al.*, 2003; Ma *et al.*, 2005; Ma *et al.*, 2006; Rasko *et al.*, 2000a; Wang *et*

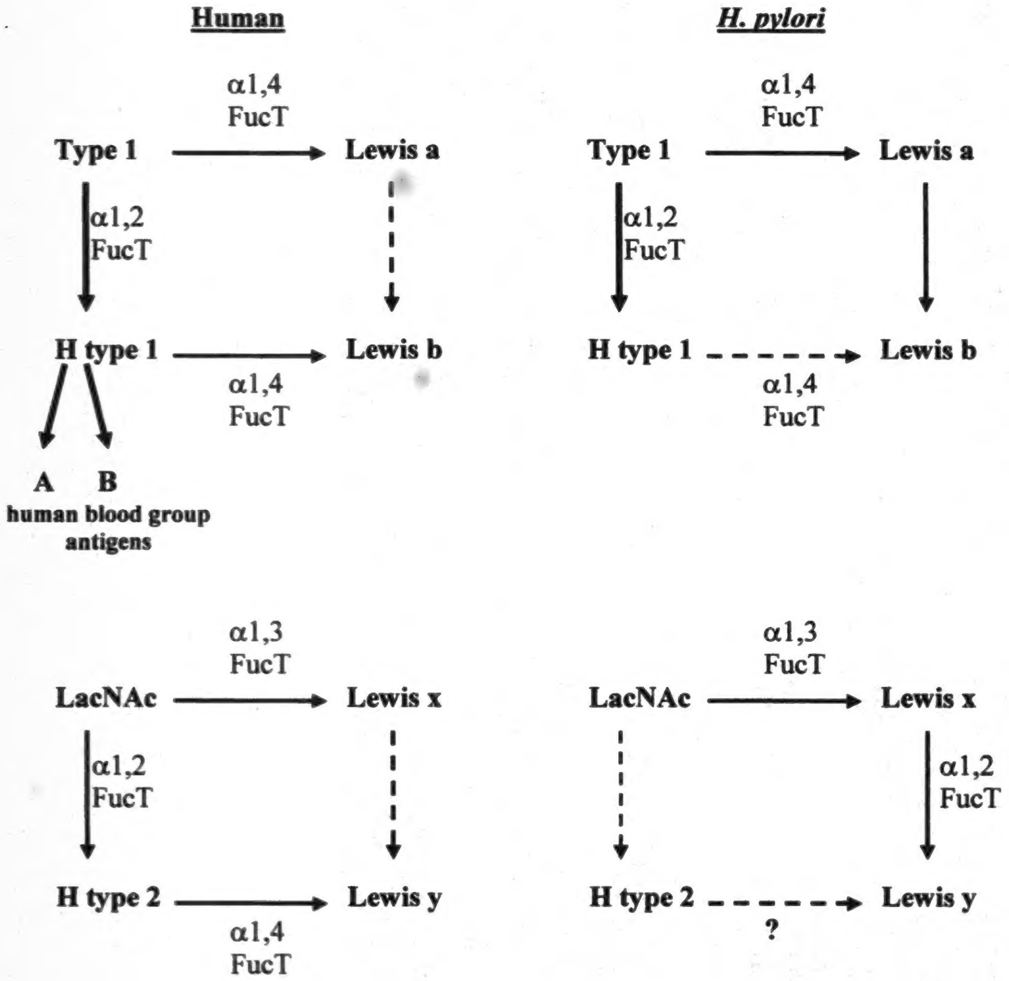

Human
H. pylori
α1,4
FucT
Type 1
Lewis a
α1,2
FucT
H type 1
Lewis b
α1,4
FucT
A
B
human blood group antigens
α1,4
FucT
Type 1
Lewis a
α1,2
FucT
H type 1
Lewis b
α1,4
FucT
α1,3
FucT
LacNAc
Lewis x
α1,2
FucT
H type 2
Lewis y
α1,4
FucT
α1,3
FucT
LacNAc
Lewis x
α1,2
FucT
H type 2
Lewis y
?

Figure 1.2 The synthetic pathways of Lewis antigens in Humans and *H. pylori*. The thickness of the arrows represents the approximate preference for the synthetic pathway indicated, based upon known enzyme activity. ? – unknown/yet to be determined pathway. Figure was adapted from Wang *et al.* 2000.

al., 1999a). All three *H. pylori* FucTs have been demonstrated to be involved in the synthesis of the Le antigens (Ge *et al.*, 1999). In contrast with the general pattern of LPS gene co-localization in other species (see 1.7.3), the *H. pylori fut* genes are not colocalized with each other or other genes involved in the synthesis of LPS structures (Fig 1.3). An interesting additional feature about these genes is that they are observed to be localized near a number of ORFs putatively involved with iron metabolism.

1.9 *Glycosyltransferase: Overview*

Glycosyltransferases (GT) are a large and diverse class of enzymes responsible for the synthesis of a huge variety of glycoconjugate structures, including glycans, glycoproteins and glycolipids. The typical GT reaction is the transfer of sugar from an activated sugar donor to an acceptor molecule (typically a lipid, protein or saccharide) with either inversion or retention of the stereochemistry at the C_1 atoms of the donor sugar (Unigil and Rini, 2000). An inverting reaction flips the configuration of the donor sugar from α to β or vice versa in an S_N2-like reaction. *H. pylori* FucTs are GTs responsible for the transfer of fucose from GDP-fucose to the terminal or subterminal end of an Galβ1,4GlcNAc (Type II, LacNAc) or Galβ1,3GlcNAc (Type I) moiety (Fig. 1.4 & 1.5). A number of mammalian GTs have been well characterized, allowing elucidation of their structure in great detail. There are now multiple crystal structures of various GTs available, although to date, there has been no published FucT crystal structure.

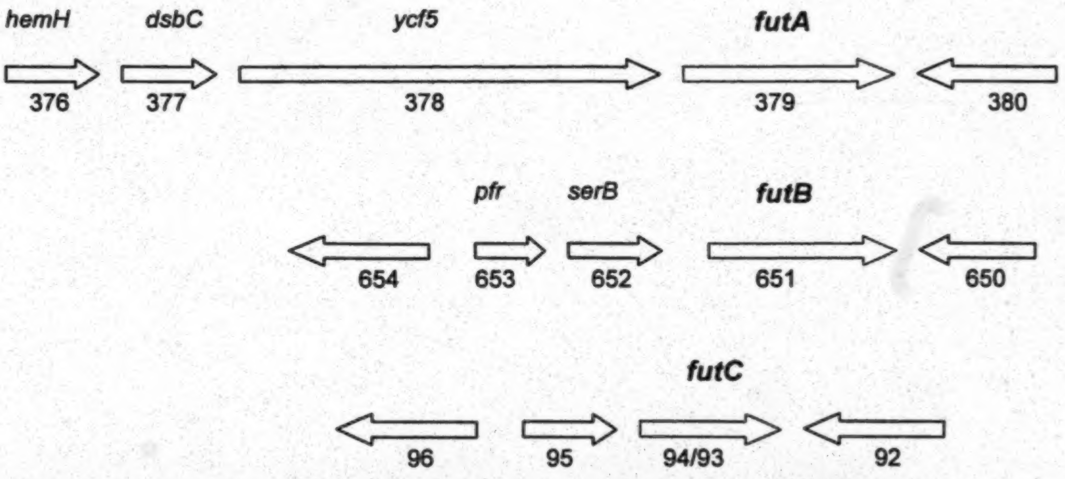
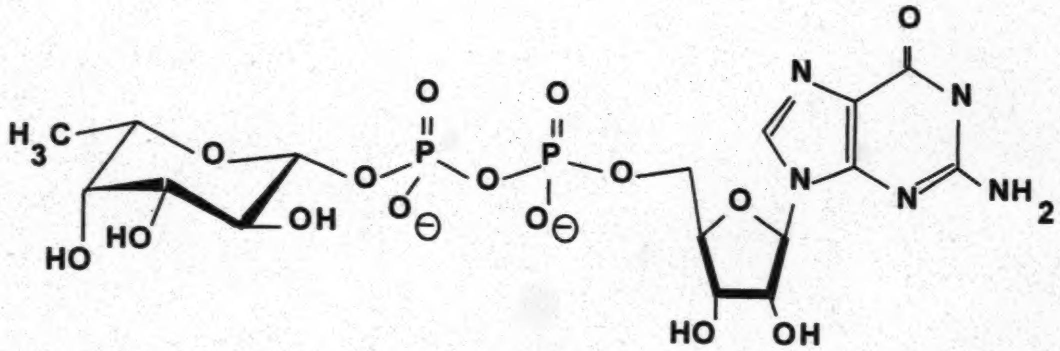

hemH
dsbC
ycf5
futA
376
377
378
379
380
pfr
serB
futB
654
653
652
651
650
futC
96
95
94/93
92

Figure 1.3 Layout of *futA, futB* and *futC* genes and their surrounding ORF's. Each arrow represents a gene in *H. pylori* and its predicted direction of transcription and approximate size. The number beneath each gene/ORF indicate its reading frame in the *H. pylori* 26695 chromosome (Tomb *et al.*, 1997). The gene names are as follows: *hemH* – ferrochelatase; *dsbC* – thiolidisulfide interchange protein; *ycf5* – cytochrome c biogenesis protein; *pfr* – non heme iron-containing ferritin; *serB*- phosphoserine phosphatases; unlabelled – unknown and/or opposite orientation to *fut* genes.



GDP-Fucose

Figure 1.4 Structure of GDP-Fucose. The nucleotide sugar donor for *H. pylori* FucTs is energized by its linkage to the nucleotide diphosphate moiety.

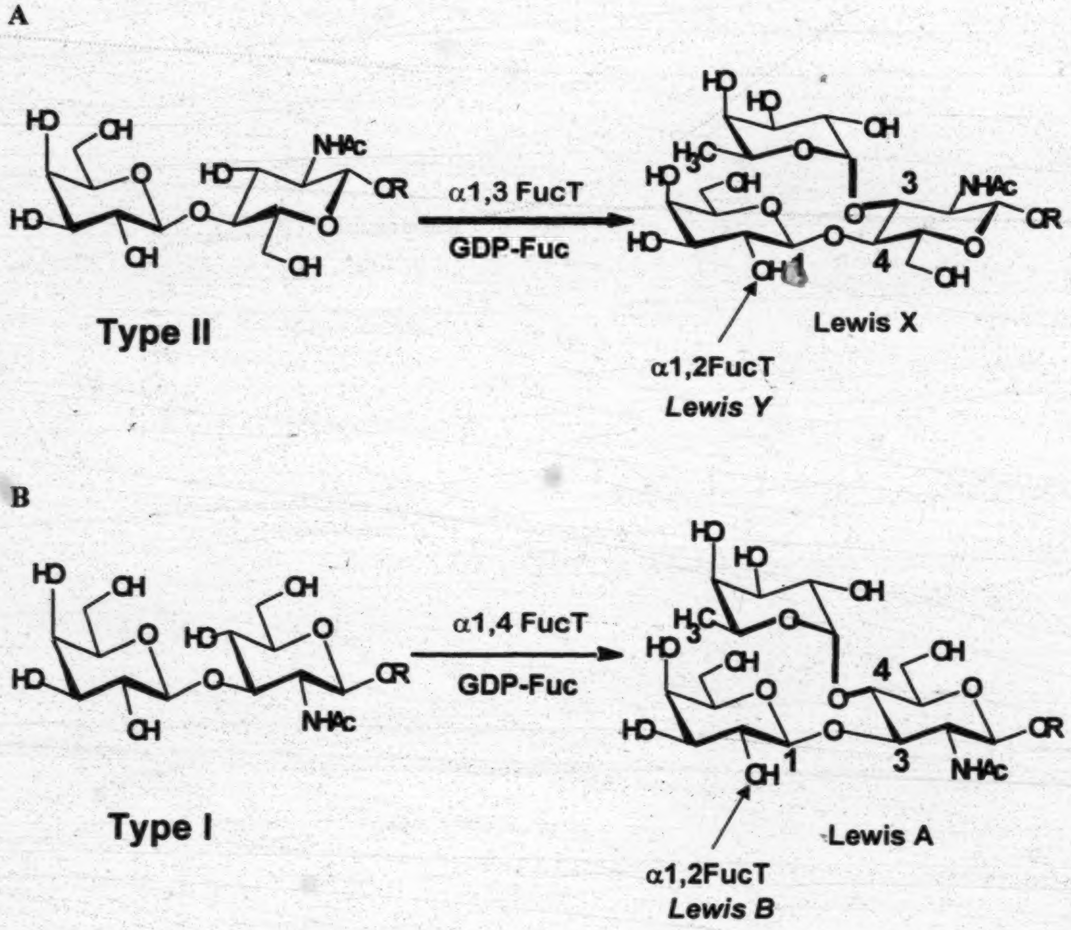

A
HO
OH
O
HO
OH
NHAc
HO
OR
O
OH
α1,3 FucT
GDP-Fuc
Type II
Lewis X
3
4
1
α1,2FucT
Lewis Y
H3C
B
α1,4 FucT
GDP-Fuc
Type I
Lewis A
α1,2FucT
Lewis B

Figure 1.5 Reactions catalysed by fucosyltransferases. A. Synthesis of Type II Lewis antigens (Le^X and Le^Y). **B**. Synthesis of Type I Lewis antigens (Le^a and Le^b).

1.9.1 Classification of Glycosyltransferases

While GT proteins show a high level of sequence divergence, there are a number of conserved structural motifs. These conserved motifs have facilitated classification of GTs into two main sub-groups, based upon the main structural folds of the enzymes (Breton *et al.*, 2006; Unligil and Rini, 2000). The first main group, Group A glycosyltransferase (GT-A) fold proteins, require a divalent metal ion for activity and are typified by N-terminal Rossmann-like domains, and C-terminal β-sheets (Unligil and Rini, 2000). The N-terminus contains the nucleotide-binding domain, and the acceptor binds at the C-terminus. A disordered loop in the acceptor domain is involved in the binding of the acceptor molecule and subsequent catalysis with the nucleotide sugar (Breton *et al.*, 2006; Yazer and Palcic, 2005). The metal dependent GT-A enzymes include most mammalian GT enzymes, as well as a number of enzymes of prokaryotic origin. The metal dependence of GT-As will be further discussed in 1.9.2.

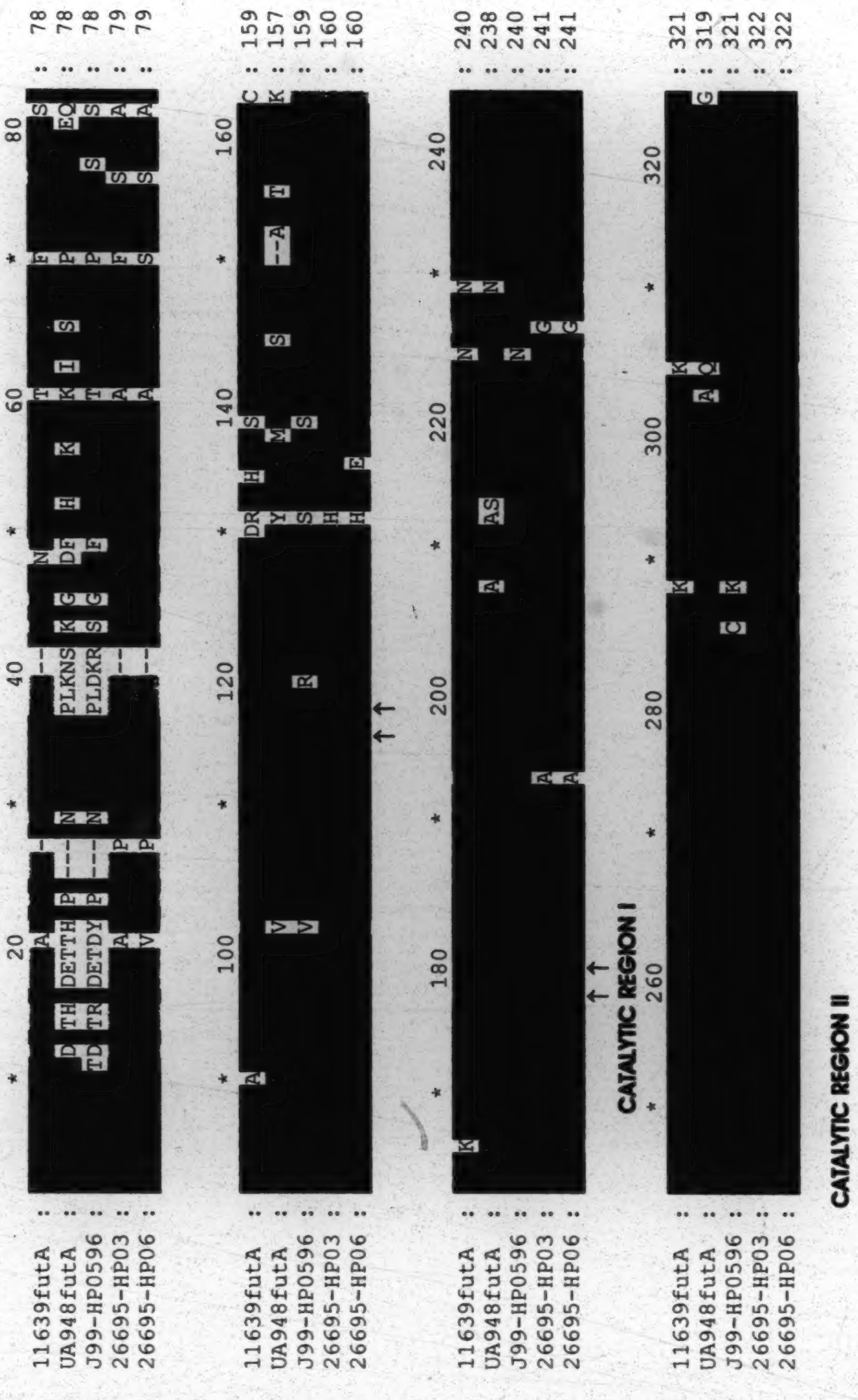
GT-B fold proteins contain two Rossmann-fold regions, separated by a cleft where donor and acceptor bind (Hu and Walker, 2002). The GT-B fold enzymes are very diverse in nature, showing a low level of amino acid sequence homology between members (Hu and Walker, 2002). Despite the variation in sequence, the crystal structures of GT-B enzymes have shown homologous enzyme topologies (Hu and Walker, 2002). The *E. coli* MurG (muramyl pentapeptide glycosyltransferase) crystal structure shares remarkable structural similarity with the two other resolved GT-B enzymes: BGT (T4 phage β-glucosyltransferase) and GtfB (glucosyltransferase from *Amycolaptosis orientalis*) (Ha *et al.*, 2000; Hu *et al.*, 2003; Mulichack *et al.*, 2001).

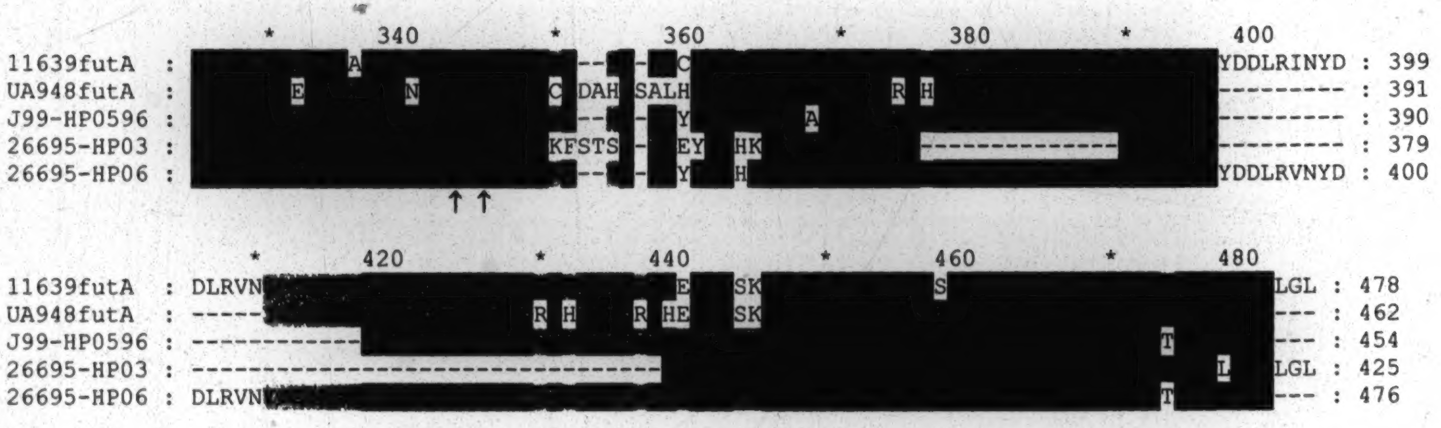
GT-C and GT-D families have also been recently created, however they are not well characterized and contain very few characterized GTs. The GT-C family contains only one crystal structure (sialyltransferase CstII from *Campylobacter jejuni*), whose structure resembles that of the GT-A enzymes, but lacks a DXD motif (Chiu, 2004). While there has been no bacterial FucT X-ray crystallographic structure determined yet, other bacterial GTs studied are found in both the GT-A and GT-B fold super-families (reviewed in Breton *et al.*, 2006).

GTs are also grouped into subfamilies according to the identity of donor and acceptor sugar (Campbell *et al.*, 1997) and peptide sequence homology in the Carbohydrate Active enZymes database (Coutinho *et al.*, 2003; http://www.cazy.org/). α1,3/4 FucTs of both bacterial and eukaryotic origin are placed in CAZy family 10, despite limited sequence homology. For instance, *H. pylori* FucTs show low sequence homology with mammalian FucTs (Rasko *et al.*, 2000a). The *H. pylori* α1,2 and α1,3 FucTs have been predicted to have a GT-B super-family structure by fold recognition models and hydrophobic cluster analysis (Breton *et al.*, 1996).

1.9.2 DXD Motif and Metal Ion Binding

One motif that has been repeatedly shown to be of significance to the activity of many GTs is the metal binding DXD motif (Boeggeman and Qasba, 2002; Tarbouriech, *et al.*, 2001; Wiggins and Munro, 1998). The DXD motif is found in both inverting and retaining GTs, and is present in the protein sequences of both GT-A and GT-B fold enzymes (Unligil and Rini, 2000). However, it is only essential for activity in GT-A fold enzymes. The function of the motif has been elucidated from crystal structures, in which the bound metal coordinates the negatively-charged phosphate groups of the nucleoside sugar donor (Boix *et al.*, 2001; Busch *et al.*, 1998). The role of this coordination is of primary importance to donor substrate binding, and the subsequent cleavage of the sugar-nucleotide bond (Boix *et al.*, 2001; Ramakrishnan *et al.*, 2004). Variants of the DXD motif, XDD and EXD are proposed to show similar coordination roles and have been demonstrated to be required for metal binding (Gulberti *et al.*, 2003). The presence of Mn^{2+} or Mg^{2+} is required for normal enzyme activity in many GTs (Charnock and Davies, 1999; Murray *et al.*, 1997; Unligil *et al.*, 2000). Notably, the nucleotide-diphospho-sugar transferase (SpsA) from *Bacillus subtilis* has been co-crystallized with both Mg-UDP and Mn-UDP, demonstrating the coordination of the metal with the phosphate groups of the nucleotide sugar which is necessary for the enzyme to be active (Chamnock and Davis, 1999). Recently, the effects of Mn^{2+} supplementation has been studied for human α1,3/4 FutIII (Palma *et al.*, 2004), and the enzyme was found to be strongly activated by Mn^{2+} addition. *H. pylori* FutA contains three conserved EXD motifs (Fig. 1.6). It is not known which, if any, of these three short motifs may be responsible for metal coordination in *H. pylori* α1,3/4 FucT catalysed addition of GDP-fucose to a LacNAc sugar backbone in the creation of the Le antigens.


11639futA
UA948futA
J99-HP0596
26695-HP03
26695-HP06
CATALYTIC REGION I
CATALYTIC REGION II



* 340 * 360 * 380 * 400
11639futA : A C YDDLRINYD : 399
UA948futA : E N C DAH SALH R H --------- : 391
J99-HP0596 : Y A --------- : 390
26695-HP03 : KFSTS EY HK --------- : 379
26695-HP06 : Y H YDDLRVNYD : 400
* 420 * 440 * 460 * 480
11639futA : DLRVN E SK S LGL : 478
UA948futA : ----- R H R HE SK --- : 462
J99-HP0596 : ------------ T --- : 454
26695-HP03 : -------------------------------- L LGL : 425
26695-HP06 : DLRVN T --- : 476

Figure 1.6 Alignment of FucT protein sequences showing the conserved EXD motifs. EXD motifs are indicated by pink font. Semi-conserved catalytic domains are indicated by blue background. The level of conservation is indicated by the shading, with the black background indicating high conservation of the indicated residue, and white background indicating low conservation of the indicated residue. (The sites of catalytic domains are discussed in Rasko *et al.*, 2000; Breton *et al.*, 1998).

1.10 *Fucosyltransferases*

FucTs are responsible for the final step in the synthesis of Le antigens, and transfer the L-fucose moiety of a GDP-fucose molecule to an acceptor molecule. The specificity of this transfer determines the Le antigen produced. α1,3 transfer typically involves transfer of GDP-fucose to a Type II acceptor, whereas α1,4 transfer is the addition of the fucose to an Type I acceptor. A number of FucTs have dual specificity and are capable of both α1,3 and α1,4 transfer. In addition, there are a number of potential sugar acceptors for either type of transfer; however, LacNAc (Type I) and Lewis C (Type II) are the most common acceptors studied. α1,2 FucTs catalyze a third type of reaction: the transfer of GDP-fucose to the non-reducing terminus site of the galactose moiety in Type I and Type II acceptor molecules.

A number of human and other mammalian FucTs have been investigated to determine residues essential to substrate specificity (Dupuy *et al.*, 1999; Dupuy *et al.*, 2004; Sherwood *et al.*, 2002). Despite the considerable interest in mammalian fucosyltransferases, surprisingly little study has been done of their bacterial equivalents. *H. pylori* FucT enzymes have been the most extensively studied prokaryote example to date. Chan *et al.* (1995) first identified a *H. pylori* FucT involved in Le^X synthesis. Since then, the Taylor laboratory has made considerable progress in characterizing both the *H. pylori* α1,3/4 FucTs (Ge *et al,* 1997, Rasko *et al,* 2000, Ma *et al.* 2003, Ma *et al.*, 2005, Ma *et al.*, 2006), and to a lesser extent the *H. pylori* α1,2 FucT (Wang, *et al.* 1999). Central to the research contained in this thesis has been further investigation of the *H.*

pylori FucTs. The commonalities and differences between human and bacterial FucTs therefore require some attention.

1.10.1 Characteristics of Mammalian Fucosyltransferases

Several α1,2 and α1,3/4 mammalian FucTs have been identified and characterized. In human tissues, nine distinct genes for FucT's that have been conclusively identified (see Breton *et al.*, 1998; Kaneko *et al.*, 1999). While there has not yet been a published crystal structure of any FucT, there has been considerable study of these enzymes to determine critical residues and their respective functions in the binding of substrates and catalysis (Dupuy *et al.*, 2004; Palma, *et al.*, 2004; Dupuy *et al.*, 1999). Human fucosyltransferase III (FucT III) is highly homologous to two other human fucosyltransferases (FucT V and VI), and exhibits dual specificity transferase activity. The FucT III is capable of both α1,3 and α1,4 transfer of fucose to the carbohydrate acceptor molecule (Fig. 1.4). Dupuy *et al.* (1999, 2004) has found that acceptor specificity of human FucT III is mediated by a single tryptophan (Trp) residue. When Trp was mutated to a non-aromatic residue, the enzyme lost its α1,4 activity and retained only α1,3 specificity (Dupuy *et al.*, 2004). In addition to the Trp residue, a highly conserved histidine-histidine motif, found towards the N-terminus, has also been found to be significant to human FucT activity in the FucT IV enzyme (Sherwood *et al.*, 2002). Mutation of the second histidine (His) residue in the motif results in increased preference for α1,3 fucose transfer to an H-type 2 acceptor (1,2 fucosylated LacNAc), relative to α1,3 transfer to LacNAc, illustrating that the His residues are involved in acceptor binding. FucT IV does not gain α1,4 activity with any His mutations, and as such it seems unlikely that these residues are involved in controlling the type of sugar transfer occurring. The FucT IV enzyme is not as strongly homologous to the FucT III as FucT V and FucT VI, however these results have indicated the significance of the conserved histidine residues to acceptor specificity. The human FucT III metal requirements have also been investigated. Palma *et al.* (2004) found the FucT III to be strongly activated by a divalent manganese ion. The manganese bound FucT III in a 1:1 ratio, and facilitated the activity by decreasing the K_M value for the acceptor substrate by 5-fold, an indication of increased affinity for the acceptor. Murray *et al.* (1996) characterized human FucT V, and found that it was also activated by manganese ions. These studies suggest that the mammalian homologues to *H. pylori* α1,3/4 FucT are metal-activated enzymes.

1.10.2 Characteristics of *H. pylori* Fucosyltransferases

Previous work from the Taylor laboratory has greatly contributed to the knowledge of *H. pylori* α1,2 and α1,3/4 FucT characteristics. The *H. pylori* α1,3/4 FucTs have been most extensively studied (Rasko, *et al.*, 2000; Ma *et al.* 2003; Ma *et al.*, 2005; Ma *et al.*, 2006). The first 80 amino acids at the N-terminal of the *H. pylori* α1,3/4 FucT have low homology between strains (~30% identity), however the conserved catalytic domains immediately following this share high identity with each other (~82%) and also show some homology with mammalian FucTs (Rasko *et al.*, 2000) (Fig. 1.6). A further unique feature of the *H. pylori* α1,3/4FucT is the presence of a variable number of heptad amino acid repeats, located near the C-terminus. The pattern of the amino acids in these sequences is DDLR(V/I)NY. While the exact function of the heptad repeats is not known, they are proposed to fold into amphipathic helices, forming a leucine zipper (Ge *et al.* 1997). These helices are predicted to play a role in potential dimerization of the protein (Ge *et al.* 1997). Recent studies in which the heptad repeats were truncated from the protein showed they are essential to activity, although partial truncations are tolerated (Ma *et al.*, 2006). However, it is likely that only in association with additional features of the protein is probable dimerization achieved (Ma *et al.*, 2006). Ma *et al.*, (2003; 2005) have used a series of elegant domain swapping and mutational experiments to elucidate the acceptor-binding characteristics of the α1,3/4FucT enzyme. Moreover, Ma *et al.* (2005) determined that mutation of a single Tyr residue at the C-terminus of the protein is sufficient to abolish α1,4 FucT activity in a dual specificity α1,3/4 FucT from strain UA948.

H. pylori α1,3/4 FucTs show low overall homology with mammalian FucTs, but have moderate levels of conservation within the two main conserved catalytic domains (Ge *et al.*, 1997; Breton *et al.*, 1998). Interestingly, the membrane association of the *H. pylori* α1,3/4FucT appears to use a topology that is the reverse of mammalian FucTs (Ma *et al.*, 2003). It seems that *H. pylori* FucTs use the C-terminal repeats and a pair of amphipathic helices to localize to the membrane in contrast to the N-terminal transmembrane helix and stalk used by the mammalian FucTs.

1.11 *Goals of Research*

The aims of this thesis were to further characterize *H. pylori* FucT expression, to determine the metal ion dependency of the α1,3/4FucT, and to characterize the transcription patterns of the *H. pylori fut* genes.

1.11.1 Metal ion Requirements of *H. pylori* Fucosyltransferase

The primary goal of these studies was to investigate the metal dependency of *H. pylori* α1,3/4FucT. As crystallization attempts to date have not been successful, mutagenesis and creative activity assay studies have been paramount to understanding the structure and function of *H. pylori* α1,3/4FucT. Mutational analysis of the three conserved EXD motifs of *H. pylori* α1,3/4FucT was performed, followed by a series of metal dependency assays, to detail the metal requirements of the bacterial FucT and to allow a comparison with human FucT requirements. This work was of particular significance because no bacterial FucT has previously been studied extensively for metal ion requirements, and very little structural information on prokaryotic FucTs is available.

1.11.2 Transcriptional Characteristics of *futA, futB* & *futC*

A second main area of investigation was to profile the gene expression patterns of the *H. pylori fut* genes (*futA, futB,* and *futC*). The organization *H. pylori fut* genes is not typical of the organization of LPS synthesis genes in other Gram negative bacteria, and there has been no study to determine whether the *fut* genes are co-transcribed with upstream genes. It was hoped that determining the operon structure of the *fut* genes would aid in better understanding of the LPS glycotype expressed by different strains of *H. pylori*.

2 Materials and Methods

2.1 *EXD Motif and Metal Binding of H. pylori α1,3/4 FucT*

2.1.1 Mutant Construction and Expression of FucT

WT and mutant α1,3/4FucT from *H. pylori* strains 11639 and UA948 were manipulated in strain *E. coli* XL1 Blue and protein expressed in *E. coli* HMS174 DE3 as previously described using a pGEM-T vector (Ma *et. al.*, 2003). Briefly, pGEM-T UA948*futA* and pGEM-T 11639*futA* in *E. coli* XL1 Blue were cultured on Luria Bertani (LB) broth agar plates containing 10 μg mL^{-1} tetracycline and 100 μg mL^{-1} ampicillin. Strains were grown and plasmid DNA isolated as per standard laboratory procedures.

FutA gene EXD acidic residues were individually substituted with alanine utilizing the QuickChange site-directed mutagenesis protocol from Stratagene (La Jolla, CA). The positions of the acidic residues mutated and sequences of the primers used for the PCR step of mutant construction are shown in Table 2.1. Primers were synthesized by Invitrogen Canada Co. (Burlington, ON) and pGEM-T vector was obtained from Promega Co. (Madison, WI). Site-directed mutagenesis PCR was performed using a high fidelity polymerase, *Pfu* turbo (2.5 U μL^{-1}) from Stratagene (LaJolla, CA). The PCR-amplified plasmid DNA was treated with 10 U *Dpn*I for 1 hr at 37 °C to digest the parental DNA strand and transformed back into *E. coli* XL1-blue host strain (Stratagene, La Jolla, CA). Each pGEMFucT mutant was sequenced by the sequencing facility in the Dept. of Biological Sciences (University of Alberta) to confirm presence of the single amino acid mutation.

Table 2.1 Primers used for site-directed mutagenesis of *H. pylori* α1,3/4FucT gene EXD motifs. The amino acid mutation is indicated in superscript in the first column. (E – glutamic acid; D – aspartic acid; A – alanine). The designations of UA948 represents the α1,3/4 FucT originating from *H. pylori* strain UA948, and the designation 11639 represents the α1,3 FucT originating from *H. pylori* strain 11639. The primer sequence is given in the 5' to 3' direction, and the bases in bold and underlined represent the mutation from the wildtype nucleic acid sequence.

Mutation	Strain	Primer	DNA Sequence (5' -> 3')	Primer Design
1st EXD Motif:				
E^{112} → A	UA948	jhFt17	CGCCATAGGCTTTGATG**C**ATTGGATTTTAACGA	UA948 E^{112} →A forward primer
E^{112} → A	UA948	jhFt18	GCGATCGTTAAAATCCAAT**G**CATCAAAGCCTATGGCG	UA948 E^{112} →A reverse primer
E^{113} → A	11639	jhFt19	CGCCATAGGCTTTGATG**C**ATTGGATTTTAATGATCG	11639 E^{113} →A forward primer
E^{113} → A	11639	jhFt20	CGATCATTAAAATCCAAT**G**CATCAAAGCCTATGGCG	11639 E^{113} →A reverse primer
D^{114} → A	UA948	jhFt21	CGCCATAGGCTTTGATGAATTGG**C**TTTTAACGATCGC	UA948 D^{114} →A forward primer
D^{114} → A	UA948	jhFt22	GCGATCGTTAAAA**G**CCAATTCATCAAAGCCTATGGCG	UA948 D^{114} →A reverse primer
D^{115} → A	11639	jhFt23	CGCCATAGGCTTTGATGAATTGG**C**TTTTAATGATCGTTA	11639 D^{115} →A forward primer
D^{115} → A	11639	jhFt24	CTCAAATAACGATCATTAAAA**G**CCAATTCATCAAAGCCTATGGCG	11639 D^{115} →A reverse primer
2nd EXD Motif:				
E^{170} → A	11639	jhFt01	GCAGTAGTGAATGATG**C**GAGCGATCCTTTGAAAAG	11639 E^{170} →A forward primer
E^{170} → A	11639	jhFt02	CTTTTCAAAGGATCGCTC**G**CATCATTCACTACTGC	11639 E^{170} →A reverse primer
D^{172} → A	11639	jhFt03	GTAGTGAATGATGAGAGCG**C**TCCTTTGAAAAGAGGG	11639 D^{172} →A forward primer
D^{172} → A	11639	jhFt04	CCCTCTTTTCAAAGGA**G**CGCTCTCATCATTCACTAC	11639 D^{172} →A reverse primer
E^{168} → A	UA948	jhFt05	CGCTAATCCATAACG**C**GAGCGATCCTTGGAAAAG	UA948 E^{168} →A forward primer
E^{168} → A	UA948	jhFt06	CTTTTCCAAGGATCGCTC**G**CGTTATGGATTAGCG	UA948 E^{168} →A reverse primer
D^{170} → A	UA948	jhFt07	CTAATCCATAACGAGAGCG**C**TCCTTGGAAAAGAG	UA948 D^{170} →A forward primer
D^{170} → A	UA948	jhFt08	CTCTTTTCCAAGGA**G**CGCTCTCGTTATGGATTAG	UA948 D^{170} →A reverse primer
3rd EXD Motif:				
E^{340} → A	11639	jhFt09	CGATTTTAG**C**AAACGATACGATTTATCACGATAACCCTTTC	11639 E^{340} →A forward primer
E^{340} → A	11639	jhFt10	GAAAGGGTTATCGTGATAAATCGTATCGTTT**G**CTAAAATCG	11639 E^{340} →A reverse primer
D^{342} → A	11639	jhFt11	CGATTTTAGAAAACG**C**TACGATTTATCACGATAACCCTTTC	11639 D^{342} →A forward primer
D^{342} → A	11639	jhFt12	GAAAGGGTTATCGTGATAAATCGTA**G**CGTTTTCTAAAATCG	11639 D^{342} →A reverse primer
E^{338} → A	UA948	jhFt13	CAAAAACATTCTTG**C**AAACGATACGATTTATCATTGCAATGATGCC	UA948 E^{338} →A forward primer
E^{338} → A	UA948	jhFt14	GGCATCATTGCAATGATAAATCGTATCGTTT**G**CAAGAATGTTTTTG	UA948 E^{338} →A reverse primer
D^{340} → A	UA948	jhFt15	CATTCTTGAAAACG**C**TACGATTTATCATTGCAATGATGCC	UA948 D^{440} →A forward primer
D^{340} → A	UA948	jhFt16	GGCATCATTGCAATGATAAATCGTA**G**CGTTTTCAAGAATG	UA948 D^{440} →A reverse primer

Expression of the α1,3/4FucT proteins was achieved as previously described (Ge *et al.*, 1997; Rasko *et al.*, 2000). *E. coli* HMS174DE3 cells were used for α1,3/4FucT expression. DE3 carries an IPTG-inducible T7 RNA polymerase. The FucT encoding *futA* has been cloned behind the T7 promoter, and has been tagged with a 6 residue histidine tag at the C-terminal end of the sequence. Cultures were transformed with pGEMFucT mutants, and grown at 30 °C with vigorous shaking (200 rpm min^{-1}) in SOC medium (20% tryptone, 5% yeast, 0.05% (w/v) NaCl, 10 mM $MgCl_2$, 2.5 mM KCl, 20 mM glucose) until an OD_{600} of ~0.3 was reached (Sambrook, 2001). Ampicillin (100 µg mL^{-1}) and rifampicin (200 µg mL^{-1}) were included for selection. Protein expression was then induced by addition of 1 mM IPTG, and cultures were allowed to continue growing for 4 hrs. The cultures were then pelleted and resuspended in HEPES buffer (20 mM, pH 7.0 containing 0.5 mM phenylmethanesulfonyl fluoride and cells lysed by three freeze-thaw cycles by immersion into liquid N_2 (-80 °C) for 2 min, followed by gentle thawing in a cool water bath. DNase (125 U ml^{-1}) and lysozyme (200 µg ml^{-1}) were added to the cell lysis mixture and incubated for 30 min on ice following the first freeze-thaw cycle. Total protein concentrations of the lysate were determined using the BCA protein assay kit from Pierce Biotech Inc. (Rockford, IL), with a bovine serum albumin (BSA) standard. This crude lysate was utilized for assessment of relative mutant activities and protein expression levels (immuno-blot analysis).

2.1.2 Immuno-blot Analysis of Protein Expression Levels

Protein expression was confirmed by immuno-blot analysis. Lysed cells were boiled for 5 min for complete protein denaturation with 4% (w/v) sodium dodecyl sulfate (SDS) and 0.002% (w/v) bromophenol blue. Samples containing equal amounts of total protein (15 μg) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (15% acrylamide, 1.4 hr at 140 V). Proteins were transferred to nitrocellulose membrane (pore size 0.22μm) obtained from Micron Separation Inc. (Westboro, MA) at 100 V for 1 hr. Membranes were blocked in PBS with 10% skim milk for 3 hrs at RT. The membrane was then probed with primary antibody (mouse anti-penta-histidine monoclonal antibody) at 1:1000 dilution in PBS with 5% skim milk overnight at 4 °C, washed 3 times in PBS and 5% skim milk for 5 min each wash, and then probed with secondary antibody (horseradish peroxidase conjugated goat antimouse IgG) at a 1:2000 dilution in PBS with 5% skim milk for 3 hrs at room temperature. The mouse anti-penta-histidine monoclonal antibody was purchased from QIAGEN Co. (Mississauga, ON) and the horse-radish peroxidase conjugated goat anti-mouse IgG was from Sigma Chemical Co. (St. Louis, MO). Blots were developed using an enhanced chemiluminescence (ECL) kit from Amersham Biosciences Corp. (Buckinghamshire, England) and the images visualized using BioMax MS film (Kodak, New Haven, CT). α1,3/4FucT band densities were determined and quantified using the Digital Science ID LE software (Kodak, New Haven CT). The expression levels of UA948α1,3/4FucT and 11639α1,3/4FucT and their respective EXD mutants were determined by comparison of their respective band densities. The enzyme activity of the α1,3/4FucT mutants was normalized to WT levels, by dividing the specific activity, mU per mg of protein, of the mutant α1,3/4FucT by the relative amount of α1,3/4FucT to account for any variation in protein expression levels.

2.1.3 Fucosyltransferase Activity Assay

For enzyme activity assays, Type II-R (βGal-(1→4)-βGlcNAc-O-$(CH_2)_8CO_2CH_3$) and Type I-R (βGal1-(1→3)-βGlcNAc-$(CH_2)_8CO_2CH_3$) acceptors were kindly provided by Dr. O. Hindsgaul (Carlsberg Institute, Copenhagen). GDP-^{3}[H]fucose (0.1 mCi mL^{-1}, 17.3 Ci mmol^{-1}) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO) and GDP-Fucose obtained from Sigma Chemical Co. (St. Louis, MO).

α1,3/4 FucT and α1,3 FucT enzyme activities were assayed as described by Ma, *et al*., (2003), with minor modifications. Briefly, the α1,3/4FucT was incubated with 200 μM donor GDP-fucose, 2 mM ATP and 0.9 mM Type II-R acceptor or 7.5 mM Type I-R acceptor in 20 mM HEPES. GDP-^{3}[H]fucose (60,000 dpm) was included in each reaction. The bovine serum albumin, and additional metal salts were eliminated from the standard reaction mixture to eliminate the influence of extraneous factors affecting the response of α1,3/4FucT metal dependence. Reverse-phase C_{18}-columns were used to isolate the trisaccharide product from the unreacted GDP-fucose. One milliunit represents the amount of fucosyltransferase activity that converts 1 nmol of acceptor substrate to product per min. The specific activity (mU mg^{-1}) was determined by dividing the enzyme activity (mU) by the total protein as determined by the standard BCA protein assay kit (Pierce). Specific activities less than 0.01 mU mg^{-1} were considered undetectable.

2.1.4 Purification of Full Length α1,3FucT

WT UA948 α1,3FucT was expressed using the pGEM vector system as previously described. To grow the large scale culture volume required, a 200 mL starter culture was grown overnight at 37 °C with vigorous shaking (220 rpm) to an OD of 1.0. The starter culture was used to inoculate 2.0 L of SOC media. The cultures were grown at 30 °C with vigorous shaking (160 rpm) until an OD_{600} of ~ 0.3 was reached. Protein expression was induced with 1 mM IPTG, and cultured for a further 5 hr. Cells were pelleted at 7000 rcf for 10 min and resuspended in 30 to 50 mL lysis solution (10 mM TRIS pH 8.0, 800 mM NaCl, 0.5 mM PMSF). Cells were lysed by three passages through the French Press at 12000 psi. Lysed cells were centrifuged at 10000 rcf for 20 min and the supernatant then filtered through 0.8 μm membrane to remove remaining cell debris.

The first Ni-ion affinity chromatography step used the automated AKTA purifier (GE Healthcare, Amersham Biosciences) fitted with a 5 mL HiTrap Chelating HP column. This method allowed for an imidazole gradient for protein elution. The second method employed a manual system, with a low pressure systolic pump and 4 mL Qiagen Ni-NTA resin. This method required a stepwise elution of the protein. Both methods followed a similar protocol for the sequence of washes and used the same buffers. Columns were conditioned with lysis buffer prior to loading the protein (10 mM TRIS pH 8.0, 800 mM NaCl). The filtered extract was then loaded onto the column, and the column washed with 20 column volumes of wash buffer (10 mM TRIS pH 8.0, 50 mM

NaCl). Protein was eluted with 20 column volumes of elution buffer (300 mM imidazole, 50 mM NaCl, 10 mM Tris pH 8.0) at a flow rate of 1 mL min^{-1}. The identity and purity of the eluted FucT was assessed by SDS-PAGE stained with Coomassie blue, and immunoblot analysis as detailed in 2.1.2.

2.1.5 Metal Dependence

Purified truncated α1,3/4FucTs (UA948$^{1\text{-}428}$ and 11639$^{1\text{-}441}$) were utilized for all metal dependence assays, so as to minimize the influence of exogenous elements such as metal salts, which would be present in the crude cell lysate. In addition, the truncated α1,3/4FucTs were preferred over full length protein for its increased solubility and increased stability following purification, making it more amenable to the incubation periods required. The removal of two putative amphipathic membrane attachment helices is thought to render these constructs less hydrophobic than WT FucT and therefore more soluble (Ma, 2006). For initial examination of the impact of EDTA on α1,3/4FucT activity, the enzyme was treated with 2.5 mM EDTA for 12 hrs at 4 °C to chelate exogenous metals in the assay mixture. Subsequent metal dependence experiments utilized a 3 hr incubation with 2.5 mM EDTA. Following treatment with EDTA, the metal ion of interest was added to a concentration of between 0 to 50 mM. The enzyme mixture allowed to reconstitute at 4 °C for 3 hrs. Standard activity assays were then performed, as described in section 2.1.3.

2.1.6 pH Dependence.

Purified truncated α1,3/4 FucT (UA948$^{1-428}$) was incubated in 100 mM citrate buffer of various pH values (range pH 3.0 – 6.0) or 100 mM HEPES buffer (range pH 7 – 8) for 3 hrs at 4 °C. Following equilibration, the other components of the assay mixture were added and enzyme activity assessed using standard procedure. The effect of the addition of additional assay components on the pH was tested and found to be minimal.

2.1.7 Mass Spectrometric Analysis of α1,3/4FucT for Metal Association

For inductively coupled plasma-mass spectrometric analysis (ICP-MS), purified α1,3/4 FucT and α1,3 FucT were prepared for analysis by dialysis, ultrafiltration using Amicon Ultra filters, or no treatment. Overnight dialysis was performed against 1 L low salt buffer (20 mM HEPES pH 7.0, 10 mM NaCl) at 4 °C to decrease the salt concentration of samples. Alternatively, buffer exchange was accomplished by ultrafiltration, using Amicon Ultra filters with 10000 molecular weight cut-off (MWCO) (Millipore, Cork Ireland), and the low salt HEPES buffer. A sample of 250 – 500 µL purified protein was loaded on the filter, and 12 mL of low salt HEPES buffer added. Protein buffer was exchanged by centrifugation of the filter at 7000 rcf for 20 min and the non-protein containing filtrate and concentrated protein fraction collected. The protein in low salt HEPES buffer was then further exchanged two more times with 14 mL low salt HEPES buffer. Presence of the protein post-exchange was verified by BCA protein assay and enzyme activity verified by activity assay. Samples were assessed by ICP-MS for presence of magnesium, manganese, and calcium. Samples were tested in two solutions:

the low salt HEPES buffer, and a low salt HEPES buffer with the addition of HNO_3 acid. The purpose of the acid addition was to increase the dissociation of tightly bound metal from the enzyme into solution. The ICP-MS was kindly performed by Zhongwen Wang, a post doctoral fellow in Dr. Le's laboratory (Dept. of Public Health Sciences, University of Alberta). The resulting counts were than analyzed following subtraction of the blank control metal counts specific to each treatment.

To better elucidate both the exact mass of the FucT protein and the possible coordination with magnesium, both electrospray and MALDI MS were attempted. To prepare the protein, purified UA948$^{1-441}$ was concentrated using Amicon Ultra filters (10000 MWCO). A sample of 500 μL to 1 mL of purified protein was loaded to filter and concentrated to 200 μL by centrifugation at 7000 rcf for 25 min. Protein buffer was exchanged as indicated above to 50 mM ammonium acetate and NaCl concentration reduced to 20 mM to minimize the presence of excess salts, which interfere with high resolution electrospray MS. Samples were submitted to the University of Alberta Department of Chemistry Mass Spectrometry facility for analysis.

2.2 *Immunofluorescence Microscopy*

The protocol for fixing and staining of cells for immunofluorescence microscopy was adapted from Hiraga *et al.* (1998). HMS174DE3 cells containing pGEM UA948FucT, or pGEM 11639FucT were induced using the method described in 2.1.1. Following protein expression, 1 mL of culture was removed and fixed by addition to 10 mL of 80% methanol at room temperature for 1 hr. Cells were then pelleted, and resuspended in 1 mL 80% methanol. A sample of 10 μL of suspended cells was allowed to adhere for 20 min to poly-L-lysine coated slides that had been prepared by applying 20 μL 0.05 g mL^{-1} poly-L-lysine in phosphate-buffered saline (PBS) and then allowed to dry for 15 min at room temperature. The cells were then treated with 100 μL of lysozyme (2 mg mL^{-1} in 25 mM Tris-HCl pH 8.0). Slides were rinsed 3 times with PBS including 0.05% Tween-20 (PBST). This was followed by a 1 min rinse with 4 mL 99% methanol and a 1 min rinse with 4 mL acetone. Slides were allowed to air dry, and then blocked overnight with 3% BSA (w/v) in PBS. Samples were incubated with 1:500 dilution of primary monoclonal antibody (mouse anti-penta-histidine) in PBST with 3% (w/v) BSA for a minimum of 2 hrs at room temperature in a moisture chamber. Slides were rinsed 5 times with PBST with 3% (w/v) BSA and incubated for 2 hr with a 1:300 dilution of secondary antibody (goat anti-mouse IgG conjugated to Alexa 488 dye). Slides were rinsed with PBST with 3% (w/v) BSA and the coverslip mounted using 60% glycerol. Slides were examined using a fluorescence and phase contrast Leica DMI 6000B microscope equipped with an Orca-ER camera (Hamamatsu, Japan). Cells were examined using 1000X magnification. To obtain images showing the localization of FucT, phase contrast images were merged with fluorescent image data using AdobePhoto CS2.

2.3 *Transcriptional analysis of H. pylori fut Genes*

2.3.1 Bacterial Strains and Media

H. pylori strains with a variety of Lewis expression phenotypes were assessed for *futA* and *futB* expression. Lewis expressing strains 26695 (Le^{XY}), 11639 (Le^{X}), G27 (Le^{Y}) and CCUG (Le^{Y}) were included. Non-typable *H. pylori* strains 25191106, PU32, and 12C2 were also used. The strains of non-typable and variable Le phenotype had been previously documented by Rasko *et al.* (2001) and Skipper *et al.* (manuscript in preparation) as having various Lewis antigens expressed on the cell surface, as assessed by ELISA and immunoblotting. *H. pylori* strains were cultured from frozen stock. The stock was thawed and 100 μL plated on BHI-YE agar plates (3.7% brain heart infusion, 0.5% yeast extract, 5% fetal bovine serum, 1.2% agar). Plates were supplemented with the following antibiotics to prevent contamination: 8 μg mL^{-1} vancomycin, 8 μg mL^{-1} amphotericin B, 0.2 % β-cyclodextrin, 5 μg mL^{-1} cefsulodin, 5 μg mL^{-1} trimethoprim, 0.2149 μg mL^{-1} polymyxin B. Plates were incubated at 37 °C under microaerobic conditions for 2-3 days. The growth of uncontaminated *H. pylori* was confirmed by urease, oxidase and catalase tests (Atlas, 1993; Koneman *et al.*, 1997). Cultures were also examined by phase contrast light microscopy to confirm motility and viability.

H. pylori was grown in liquid culture in BHI-YE) with a pH of 7.0 as adjusted with HCl. Media was supplemented with the same antibiotics used in solid media. Liquid culture of *H. pylori* was initiated by inoculating with 1:50 dilution of an overnight starter culture of *H. pylori*, in exponential phase growth and grown at 37 °C overnight with agitation (150 rpm) in microaerobic conditions.

2.3.2 RNA Isolation

Total RNA was isolated from *H. pylori* strains 26695, 11639, G27, 25191106, 12C2 and PU32 using a total RNA extraction kit (RNeasy-mini, Qiagen, Mississauga, ON.), after the bacterial pellet was allowed to lyse in 5 mg mL^{-1} lysozyme for 10 min. Following RNA elution from RNeasy columns, RNA was treated with DNase (109 U μL^{1}) at 37 °C for 20 min. Purity was assessed by heating to 68 °C for 8 min and separation by gel electrophoresis (1.2% agarose for 45 min at 110V) followed by staining with ethidium bromide and visualization under UV light. The RNA was quantified by absorbance at 260 nm.

2.3.3 RT- PCR

RT- PCR was performed using a Superscript III RT kit (Invitrogen), using a 20 µL final reaction volume and 2 µg of total RNA. dNTPs (1 mM) and *futA* or *futB* gene specific primer (1 µM) were incorporated into the reaction. The reactions were hybridized for 1 hr at 45 °C and cDNA synthesized at 50 °C for 3 hrs. Following synthesis, each 20 µL volume of cDNA supplied 10 PCR amplification reactions.

The cDNA was PCR amplified using primer pairs specific for adjacent genes (Table 2.2). Primers were synthesized by Invitrogen Canada Co. (Burlington, ON). Standard PCR was performed using Taq provided by Invitrogen. These primers were designed using the 26695 and J99 annotated sequences accessed from TIGR Comprehensive Microbial Database (www.tigr.org). The assay mixture for amplification included cDNA template (2.0 µL), 1 µM of each gene specific primer, 3 mM $MgCl_2$, and 4 mM of each dNTP with standard Taq buffer. Amplification used the following program: pre-incubation at 94 °C for 2 min, denaturing at 94 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 65 s for each kb of target sequence for 30-35 cycles. Negative (no Superscript III enzyme, and no Taq) and positive controls were included in all PCR amplifications.

2.3.4 Visualization and Analysis of Data

The PCR products were analyzed by agarose gel electrophoresis, and sequence analysis by Dept. of Biological Sciences sequencing facility (University of Alberta).

Gels were stained with ethidium bromide and PCR product size determined relative to a 1+ Kb DNA ladder from Fermentas (Burlington, ON). Following agarose gel electrophoresis visualization, the target genes were considered to be co-transcribed when the PCR product matched the expected size

Table 2.2 Primers used for RT-PCR amplification of *fut* gene transcription studies. The DNA sequence is given in the 5' to 3' direction. The desired gene product is indicated by the right most column with the orientation of the primer indicated in brackets. The putative gene names are as follows: *hemH* – ferrochelatase; *dsbC* – thiolidisulfide interchange protein; *ycf5* – cytochrome c biogenesis protein; *pfr* – nonheme iron-containing ferritin; *serB*- phosphoserine phosphatase; *ureA* – urease A; upper – coding strand; lower – anticoding strand

Primer	Sequence (5' → 3')	Melting Point (°C)	Product Size (bp)	Desired Gene Product (primer position)
***futA* operon region**				
0104	GCT CTC GTG ATC TTG GCT TAT TTC	55.4	231	*yct5* (upper)
0404	TTG GTG GAG GGT GAT TGC GTA	57.5		*futA* (lower)
1204	TTT ATG GCG TTT GAT TTC TGC TAT	54.2	501	Upstream (upper)
1304	TTT AAA AAC ACC CCC ACT TCA T	52.1		*hemH* (lower)
1404	ATT GAT GCC GGC GAT ACT TAC CAG	60.1	723	*hemH* (upper)
1504	CTT TCG CAA TAG GAG CAA CCA T	55.1		*dsbC* (lower)
1604	ATA AAA CCG GCA AAA CCA TCT	52.2	266	*dsbC* (upper)
1704	ATA GAG TGC GAT TAA AGG GAT AGC G	56.3		*yct5* (lower)
***futB* operon region**				
0804	TTG GTG GAG GGT GAT TGC GTA	57.5		*futB* (lower)
0904	CGA TGG GGC GAA TGA CTT GA	58.0	360 (with primer 0804)	*serB* (upper – short product)
1004	TGA AAG CTA TGA ATG GCG AGA CAG	57.4	739 (with primer 0804)	*serB* (upper – long product)
1104	TGC AAT GGT ATG TGT CTG AAC AGC	56.5	1008 (with primer 0804)	*pfr* (upper)
1804	TCA AAC CTT CAA ACT TAT GCT CAG	52.4	725	Upstream (upper)
1904	ACT TCT TTA ATC CCC TTG TTG TAG	50.1		*pfr* (lower)
Positive control				
ureA F	ATGAAACTCACCCCAAAAGAG		714	*ureA* (HP0073) *
ureA R	CTCCTTAATTGTTTTACATA			

* Primer design of J2104 and J2004: Harris, A.G., *et al.* (2002).

3 Results

3.1 *Metal Dependence of FucT*

3.1.1 EXD Alignment

Alignment of the α1,3 and α1,3/4 FucT protein sequences from a number of different *Helicobacter pylori* strains reveals that three EXD motifs are well conserved (Fig. 1.4). This suggests that these short amino acid motifs may be significant to the catalysis promoted by FucT. DXD/EXD motifs typically fall within a sequence flanked on either side by apolar residues (h) in the following pattern: hhhhDxDxh, with x indicating any amino acid residue (Wiggins and Munro, 1998). When the sequences of glycosyltransferases from different families are compared, it is also observed that the third of these four hydrophobic residues that precedes the DXD is typically an aromatic amino acid (Unligil *et al.*, 2000). Analysis of the *H. pylori* FucT alignment (Fig. 1.4) shows that the first and second EXD motifs most closely follow this pattern in the FucT of both UA948 (α1,3 and α1,4 activity) and 11639 (α1,3 activity). As such, it was expected that one of these motifs was most likely involved in coordinating a metal ion required for catalysis.

3.1.2 EXD Mutant Activity Analysis

The Glu and Asp residues in all three EXD motifs were mutated in *futA* from UA948 and 11639 cloned into the pGEM vector system. The presence of the intended mutations in *futA* from these two strains was verified by sequencing. Expression of α1,3/4 FucT was induced at 30 °C in SOC media with the addition of 1 mM IPTG for 4 hrs. The expression of the mutant FucT protein in *E. coli* confirmed by immuno-blot (Fig

3.1) by probing using anti-penta-histidine monoclonal antibody which recognizes the his tag added to all FucT's. The α1,3 and α1,4 fucosyltransferase activity of the FucT's with E→A or D→A mutations were assessed and compared to that of the WT (Fig. 3.2). This allowed for standardization of activities to relative protein expression levels. The α1,3 and α1,4 activities of each mutant were determined using Type II acceptor (0.9 mM) and Type I acceptor (7.5 mM), respectively. The specific activity of α1,3 fucosyltransferase activity for WT UA948 FucT was 26.59 ± 5.19 mU mg^{-1} total protein and for WT 11639 FucT was 15.17± 4.33 mU mg^{-1} total protein. The specific α1,4 transferase activity in UA948 FucT was 1.42± 0.56 mU mg^{-1}, while 11639 FucT had no measurable α1,4 activity. The activities of the FucT mutants are shown as a percentage of the WT activity of the appropriate strain. All EXD mutants, in both the α1,3/4 FucT (strain UA948) and the α1,3 FucT (strain 11639) showed significantly reduced activity (Fig. 3.2). This is not unexpected, as acidic amino acid residues are typically quite important to protein secondary structure. The residue that displayed the most critical significance to the activity of both 11639 FucT and UA948 FucT was the glutamic acid residue in the first motif (UA948 E^{112} and 11639 E^{113}). It is interesting to note that FucT activity was not completely abolished in any of the mutants constructed; even the most deleterious mutation was not found to be absolutely critical to FucT activity. This suggests that glutamic and aspartic acid residues in the FucT EXD motifs are not essential for activity.

The effect of the EXD mutations was similar on α1,3 and α1,4 activity (Fig. 3.2) of the dual specific UA948 FucT. It appears the glutamic and aspartic acid residues are of equivalent importance to the α1,3 and α1,4 activities of the enzyme. This is

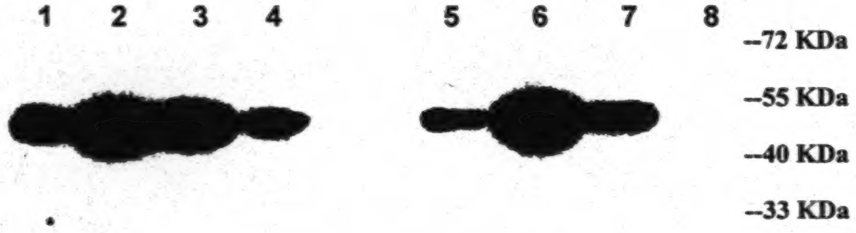


Figure 3.1 Representative immunoblot of wild type UA948 α1,3/4 FucT and six single amino acid mutants from UA948FucT expressed in *E. coli.* The FucTs with His_6 tag were expressed in *E. coli* HMS174DE3 cells with induction using 1 mM IPTG and growth at 30°C for 4 hrs. The FucT proteins were detected with mouse anti-penta-histidine monoclonal antibody. 15 μg of crude cell lysate was loaded in each lane. The band densities were determined using Digital Science ID LE software. Lane 1: UA948FucT $E^{112} \rightarrow A$; Lane 2: UA948FucT $D^{114} \rightarrow A$; Lane 3: UA948FucT $E^{168} \rightarrow A$; Lane 4: UA948FucT $D^{170} \rightarrow A$; Lane 5: UA948FucT $E^{338} \rightarrow A$; Lane 6: UA948FucT $D^{340} \rightarrow A$; Lane 7: UA948FucT Wildtype; Lane 7: Lane 8: pGEM vector alone

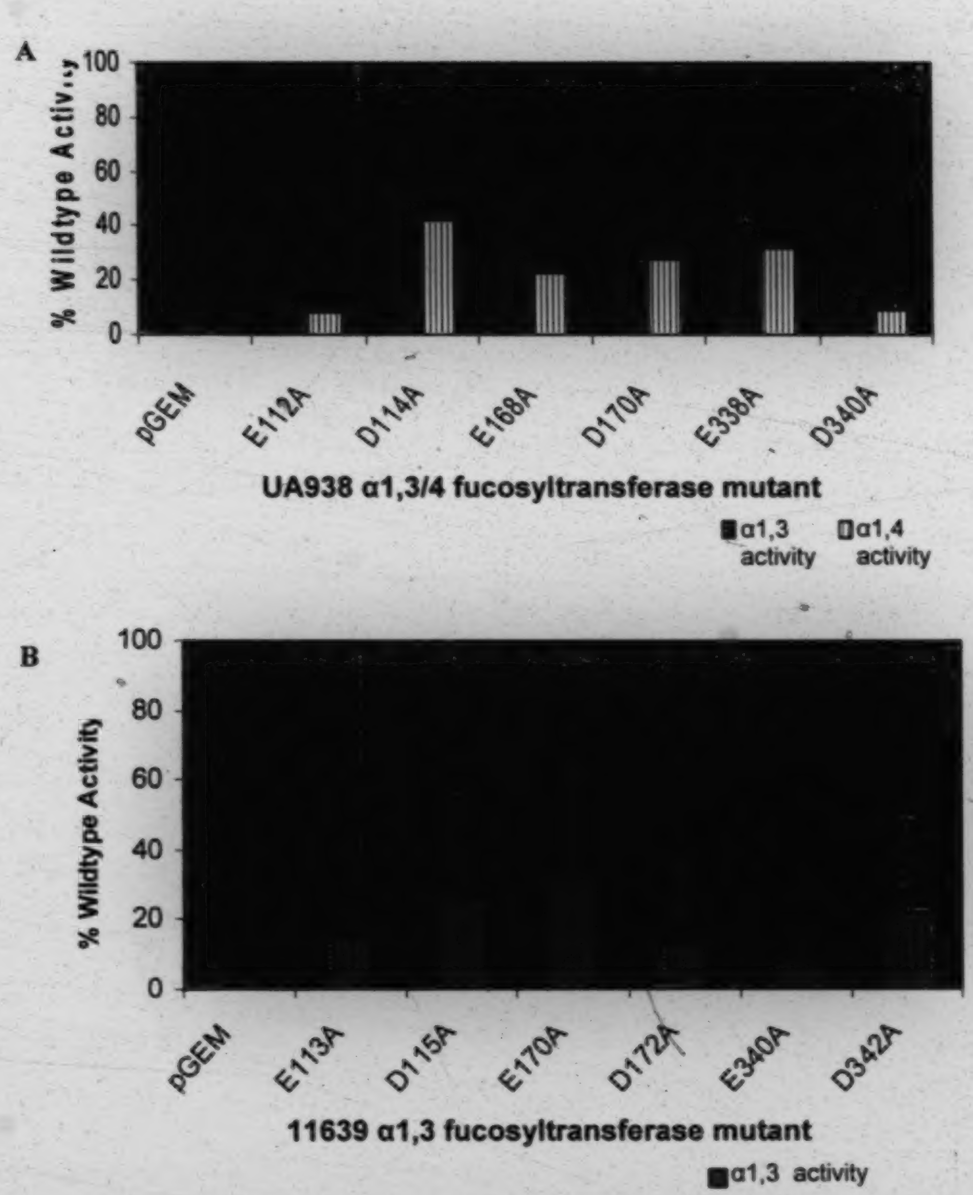


Figure 3.2 Relative fucosyltransferase activities of FucT EXD mutants. Fucosyltransferase activities of the point mutants are compared relative to wildtype activity levels (wildtype activity set to 100%). The number designates the amino acid position that was mutated. pGEM represents the negative vector control, which does not express the fucosyltransferase. The α1,3 and α1,4 activities of each mutant were determined using Type II acceptor (0.9 mM) and Type I acceptor (7.5 mM), respectively. **A**. Relative α1,3 and α1,4 fucosyltransferase activity of UA948 α1,3/4 FucT EXD motif mutants; **B**. Relative α1,3 activity of 11639 α1,3 FucT EXD motif mutants. Each value represents the average of three to four determinations with the standard deviations indicated.

interesting, as previous characterization by our laboratory has demonstrated that the acceptor specificity of FucT for α1,4 activity can be determined by a single amino acid residue at the C-terminus of the enzyme (Ma *et al.*, 2005). The results from the current mutational studies suggest acceptor specificity of the enzyme is not affected by the possible disruption to the sugar-nucleotide binding site. This is consistent with the findings of Ma *et al.*, indicating that acceptor and donor binding may occur independently of one another in α1,3/4 FucT.

Acceptor kinetics were performed on the most deleterious mutants (UA948 E^{112}; 11639 E^{113}) and compared to that of WT (Table 3.1). Kinetic analysis of the acceptor did not aid in further determination of the significance of a potential metal binding motif. The mutation activity analysis of the *H. pylori* α1,3/4 FucT suggested that the enzyme does not require coordination to a metal ion through a DXD motif. A lack of dependence upon metal ions is predominant in glycosyltransferases in the GT-B fold families, and is one of the fundamental differences between the GT-B and GT-A families (Hu and Walker, 2002); however, the metal dependence of *H. pylori* α1,3/4 FucT has not been previously studied. Thus, these data prompted a closer investigation of *H. pylori* FucT metal requirements.

3.1.3 Purification Trial on Full Length FucT

Our lab has previously determined that *H. pylori* FucT is likely a membrane-associated protein (Ma *et al.*, 2003), and therefore would not be amenable to purification as a full length WT protein for structural studies. An efficient purification protocol has been developed for a soluble FucT construct with 34 ($UA948^{1-428}$) or 37 amino acids (11639^{1-441}) deleted from the C terminus (Ma *et al.*, 2006). While these truncated FucTs

Table 3.1 Acceptor kinetics of wildtype fucosyltransferase and most deleterious EXD mutants constructed (11639 $E^{113}A$, UA948 $E^{112}A$). Acceptor kinetics were determined using 0.03–2 mM Type II-R, or 0.4–25 mM Type 1-R with GDP-Fuc at 200 μM and GDP-^{3}[H]Fuc at 0.2 μM. NA – not applicable.

	Type II-R[a]			Type I-R[b]		
Enzyme	**K_m (mM)**	**V_{max} (mU/mg)**	**V_{max}/K_m**	**Km (mM)**	**V_{max} (mU/mg**	**V_{max}/K_m**
11639 FutA	0.28 ± 0.09	6.30 ± 0.13	6.48	NA	NA	NA
11639 $E^{113}A$ FucT	1.2 ± 0.27	2.37 ± 0.08	1.24	NA	NA	NA
UA948 FutA	2.0 ± 0.48	8.68 ± 0.10	3.88	8.2 ± 1.2	1.92 ± 0.10	0.86
UA948 $E^{112}A$ FucT	0.4 ± 0.15	0.25 ± 0.03	0.54	6.4 ± 1.3	0.17 ± 0.03	0.37

a. Type II-R: Galβ1,4GlcNAc-O(CH2)CO2CH3 (α1,3 activity)
b. Type I-R: Galβ1,3GlcNAc-O(CH2)CO2CH3 (α1,4 activity)

have both increased solubility and stability following purification, there is also unfortunately a significant reduction in fucosyltransferase activity. UA948$^{1-428}$ activity is most significantly affected by the truncation, with specific transferase activity dropping to approximately 20% of WT levels. An additional limitation of this protocol is the decreased level of expression of these constructs in the HMS174DE3 expression system.

The goal of the full length purification was thus two-fold: firstly to attempt to obtain WT purified enzyme for further investigation of metal dependence, and secondly, to develop an alternative purification protocol for use in further protein characterization such as crystallization studies. For crystallization work, it would be valuable to have a full length protein to work with, as any structure obtained would then comprise all the details of the WT protein. The α1,3 FucT from *H. pylori* strain 11639 was chosen for this study, as it proved to be the more stable of the two enzymes (Ma *et al.*,2006). Two types of Ni^{2+}-columns were used for purification of the His-tagged 11639 α1,3 FucT, the Amersham Pharma via Hi-trap Ni^{+} resin on an AKTA automated system and the Qiagen Ni-NTA Superflow resin on a low pressure manual system. The rationale for attempting two systems was based on the high sensitivity of the FucT to elution from Ni^{2+}-columns, and the previous findings that glycosyltransferases are on occasion more amenable to step-wise manual elution purification schemes over automated gradient systems (Palcic, M., personal communication). The AKTA purification resulted in a lower level of purification but the enzyme retained good activity levels (Fig. 3.3). The low pressure system, using the Qiagen His-Trap chelating resin resulted in a purer elution, but lower levels of activity (Fig. 3.4). The majority of the protein loaded did not bind to the resin

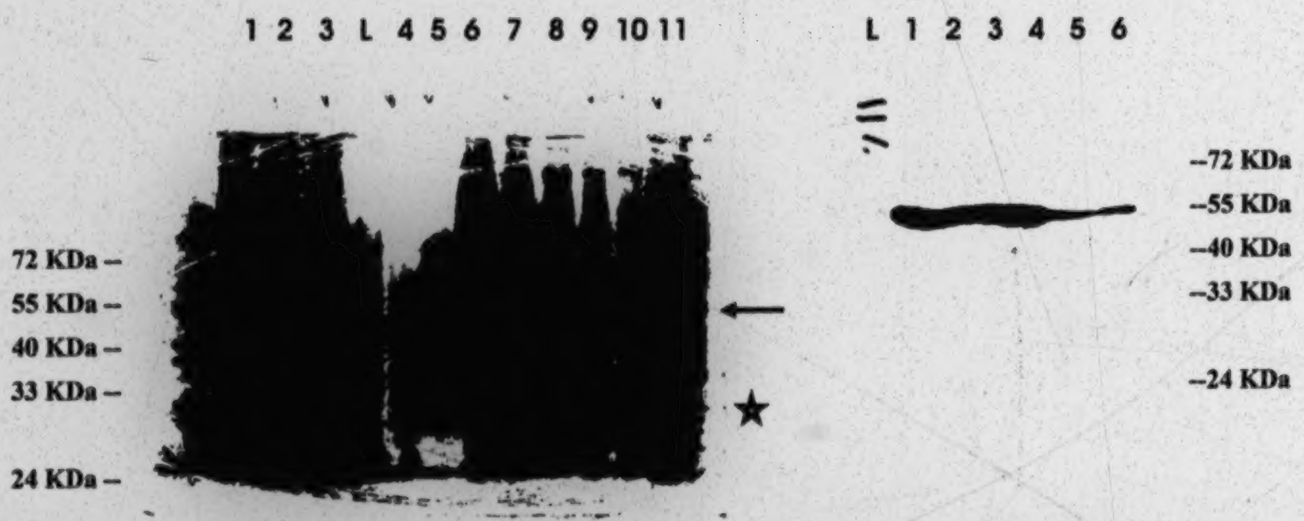


Figure 3.3 Analysis of purification of full length 11639 α1,3/4FucT using the AKTA system with HiTrap Chelating Resin. Level of purification obtained for full length FucT using the AKTA system with Ni-affinity column and an imidazole gradient elution. **A.** SDS-PAGE separation of purification fractions, as labeled. Gel was stained with Coomassie blue. The arrow indicates the location of FucT. The star represents the location of the break down product was observed. Lane 1 - pre French press suspension; Lane 2 – French pressed cell supernatant; Lane 3, 11 – Flow through; Lane 4 – 10 – Sequential elution fractions; Lane L - Ladder. **B.** Immunoblot of purification fractions, as labeled. α1,3/4 FucT with His_6 tag was detected using mouse anti-penta-histidine monoclonal antibody. Lane 1 – Flow through; Lane 2 – 4 – Sequential elution fractions; Lane 5 - pre French press suspension; Lane 6 - French pressed cell lysate.

and was found in the flow through. Only 6% of the total protein loaded was collected during the elution stage of the purification. The low level of binding to the column may be indicative of a number of factors: the capacity of the Qiagen Ni-NTA resin to bind protein, the low solubility of full length FucT, or an affinity problem due to a partially obstructed histidine-tag. The first possibility is unlikely, as the bacterial cell membrane would have been lost during the French press and centrifugation steps prior to the application of the lysate to the column. It is more probable that much of the FucT protein was flushed from the column immediately due to poor affinity of the FucT histidine-tag. Coomassie-blue stained SDS-PAGE gels demonstrate the increased level of purity attained by both purification methods, but also show the susceptibility of the α1,3 FucT to proteolysis (Fig. 3.3 & 3.4). The predicted molecular mass of the α1,3 FucT is 56.0 kDa. The SDS-PAGE gels show a significant band at a mass just below 55 kDa. The suspected proteolysis product appears at a mass below the 33 kDa marker, and elutes after the full length FucT. The purified protein eluted from the Qiagen Ni-NTA resin showed an increase in specific activity from 0.9 mU mg^{-1} to 3.7 mU mg^{-1} (Fig. 3.5). The protein purified by this method was very susceptible to proteolysis on the column resin, and thus it was decided to use purified truncated FucT constructs for the metal and pH dependence studies. This construct of the FucT enzyme lacks two putative α-helices at the C-terminal end to assist in solubility. Details of purification of these constructs, UA948$^{1\text{-}441}$ and 11639$^{1\text{-}428}$ FucT, may be found in Ma, *et al.* (2006). The purification work with the full length protein is valuable, as it demonstrates the potential feasibility of this purification for future structural studies, although this approach would require very careful attention to the integrity of the protein throughout the process.

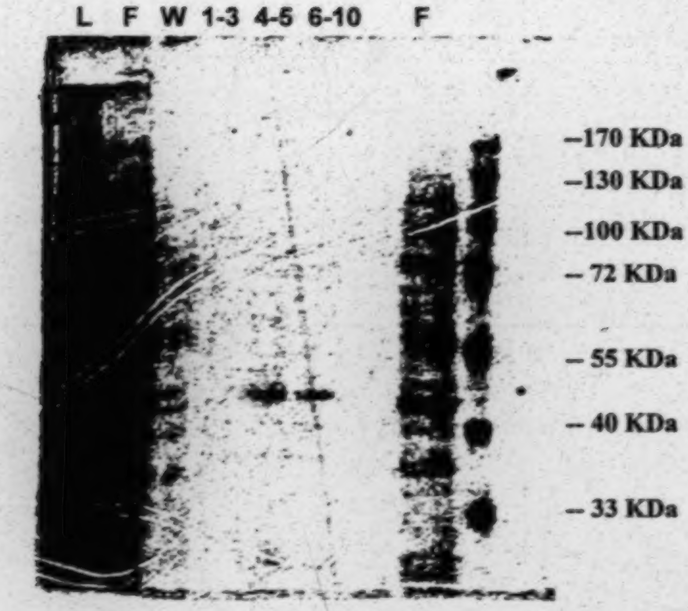
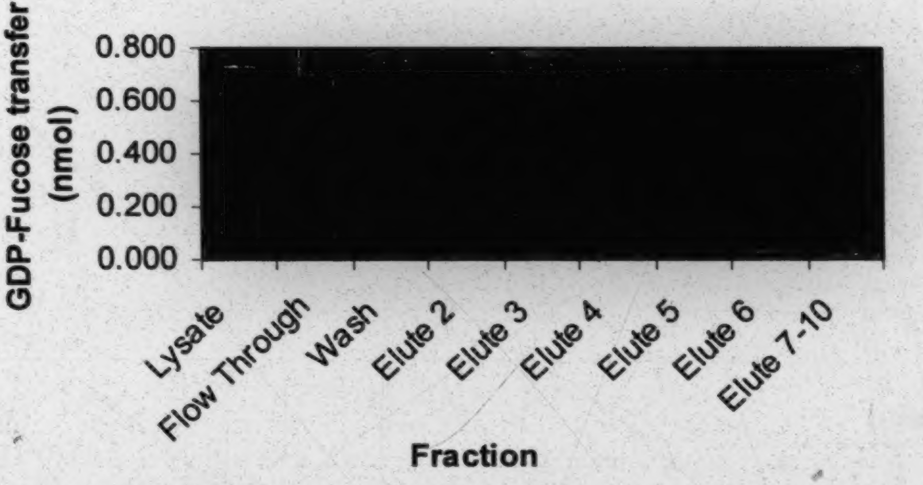

L
F
W
1-3
4-5
6-10
F
–170 KDa
–130 KDa
–100 KDa
– 72 KDa
– 55 KDa
– 40 KDa
– 33 KDa

Figure 3.4 Coomassie blue-stained SDS-PAGE gel of UA948 α1,3/4FucT purified using low pressure manual system with Ni-NTA resin and stepwise elution. L – Lysate loaded to column; F- Flow through; W- Wash elution; 1-3, 4-5, 6-10 – Combined elution fraction numbers.

A.



B.

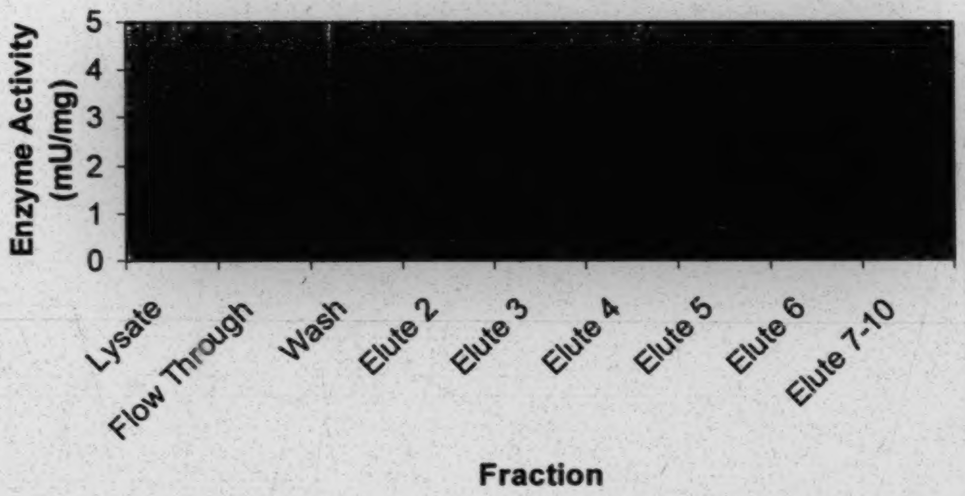


Figure 3.5 Purification profile of FucT activities using manual low pressure system with Ni-NTA resin and step-wise elution. A. The total GDP-fucose (nmol) transferred in the reaction mixture, as followed through the purification process. **B.** The specific fucosyltransferase activity (mU mg^{-1}), as followed through the purification. Values are from a typical elution, as there was variation in the amount of protein loaded and eluted between purification trials.

3.1.4 Influence of Divalent Cations on FucT

To further elucidate the metal requirements of the *H. pylori* α1,3/4 FucT, and confirm that it is not reliant on metal binding, a series of assays were performed in which the exogenous metals were removed from the reaction mixture and α1,3/4 FucT, and then selectively reconstituted with divalent metal ions. The purified truncated form of the FucT (UA948$^{1\text{-}428}$ and 11639$^{1\text{-}441}$) were used for these assays, so as to minimize any interference from exogenous elements present in the cell lysate. Activity levels of the purified constructs are lowered from the WT enzyme level, with specific activities between 5 - 10 mU mg^{-1}. To determine if α1,3/4 FucT had metal independent activity, purified α1,3/4 UA948$^{1\text{-}428}$ FucT was treated with a range of EDTA concentrations for a 12 hr incubation on ice following which activity assays were performed (Fig. 3.6). These assays showed that *H. pylori* FucT retained high levels of activity in the absence of any exogenous metals, although fucosyltransferase activity was depressed 25 - 40% below that of WT levels in a typical assay. This may suggest that EDTA treatment does strip activating metal ions from the WT enzyme, thereby depressing its activity. When the length of a 2.5 mM EDTA incubation was shortened to 3 hrs, the impact on the α1,3 FucT activity was found to be comparable. For experimental efficiency, the incubation times was thus shortened for the metal dependence assays.

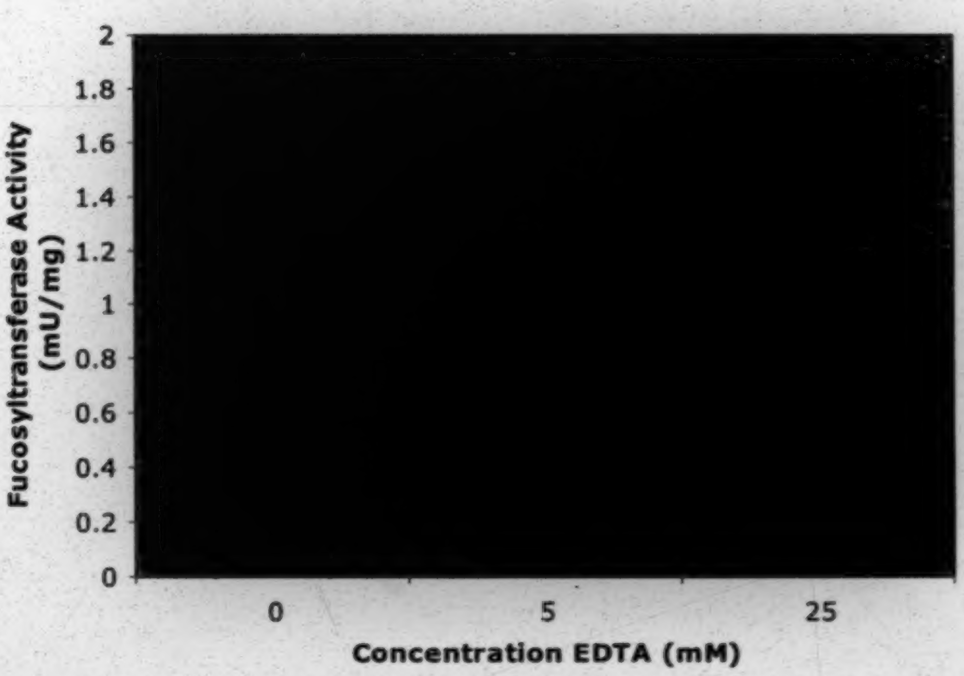


Figure 3.6 The effect of EDTA treatment on UA948$^{1\text{-}428}$ α1,3 FucT activity. The concentration of EDTA is indicated on the X-axis, and was incubated with the fucosyltransferase for 12 hrs at 4°C prior to assessment of activity. A representative sample of results is shown.

In the initial trial to further elucidate the influence of metal ion addition on enzyme activity, α1,3/4 FucT was first treated with EDTA and then dialyzed overnight to remove exogenous EDTA and metal prior to the addition of a panel of metal ions and activity assays. Since the use of dialysis to remove EDTA was not found to increase activity of the enzyme on addition of metal ions, this step was subsequently eliminated, so as to prevent loss of activity due to prolonged incubation before assessing activity. The results in Fig. 3.7 show the effect of divalent cation supplementation on α1,3/4 FucT activity relative to an EDTA treated α1,3/4 FucT control. Interestingly, the majority of cations were strongly detrimental to α1,3/4 FucT activity. Only Mg^{2+}, Ca^{2+} and Mn^{2+} supplemented enzyme maintained substantial levels of activity. In addition, a high level of variability was noted in the enzyme activity levels between trials. The relative lack of inhibition of FucT activity by Ca^{2+} was unexpected. Ca^{2+} has not been documented as a co-factor for other GT's activity. A possible explanation of the Ca^{2+} influence is that, as the preferential chelator of EDTA, it binds to and displaces other metal ions from the EDTA molecules, thereby liberating the other metal ions to bind to FucT. In this panel of cations, it was also worth noting that Mg^{2+} had the strongest activating effect on FucT activity increasing the activity by over 50%. This was further studied in the experiments of section 3.1.5.

3.1.5 Comparison of the Influence of Mg^{2+} and Mn^{2+} on FucT Activity

The initial survey panel of metal ion influence on α1,3/4 FucT was followed by a more detailed comparison of the influence of Mg^{2+} and Mn^{2+} on enzyme activity. Only

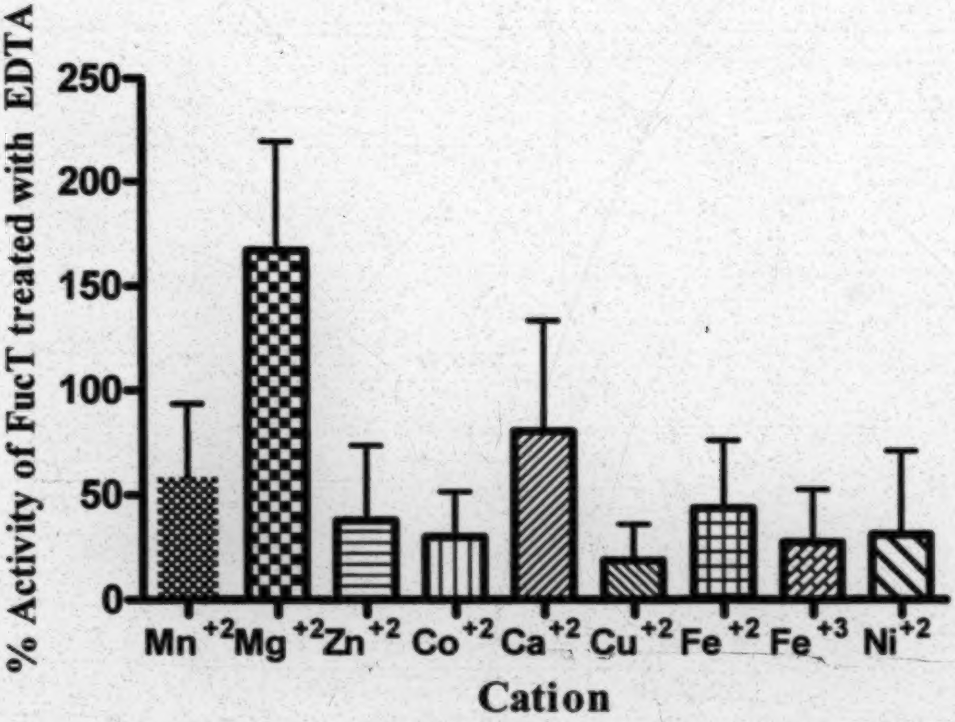
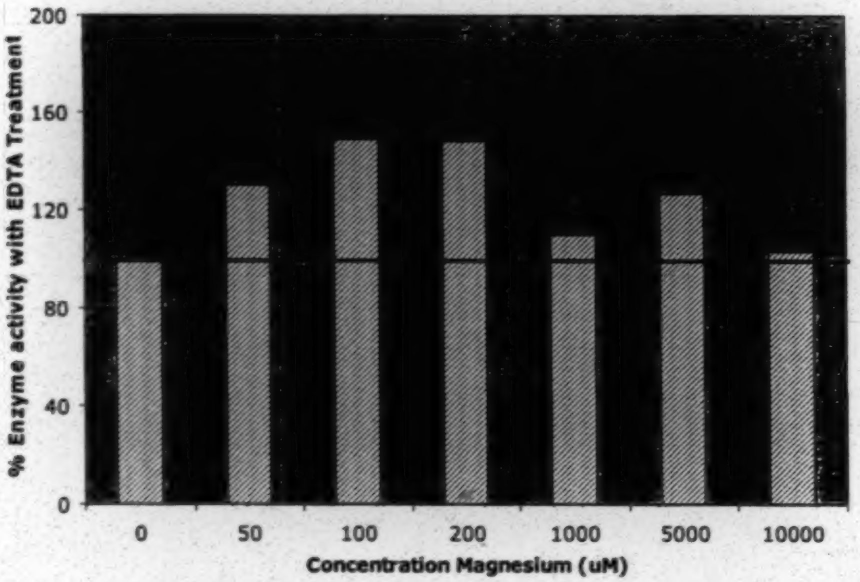


Figure 3.7 Influence of metal ions on EDTA treated purified UA948$^{1-428}$ α1,3 FucT activity. Following treatment with 2.5 mM EDTA for 3 hrs at 4°C, 25 mM of the indicated metal cation was supplemented to the reaction and incubated for 3 hrs at 4°C. The influence of metal ion supplementation on purified UA948$^{1-428}$ α1,3 FucT activity was assessed relative to the activity of UA948$^{1-428}$ following EDTA treatment. Values represent an average of a minimum three independent experiments, with the standard deviation represented by the error bars.

Mg^{2+} was found to consistently serve as an activator for FucT (Fig. 3.8). The highest level of activation was observed with a moderate excess of Mg^{2+} of 100 – 200 μM, as compared to the protein concentration (5.4 μM). Concentrations exceeding 200 μM ceased to positively impact α1,3/4 FucT activity. The observation that Mg^{2+} was the sole metal ion capable of activating the α1,3/4 FucT was unexpected, and contrary to what has been observed for the majority of glycosyltransferases whose activity is dependent on Mn^{2+} (Hu and Walker, 2002). The results shown are variable between trials, but consistently demonstrated the same pattern of activation. Mn^{2+} appeared to be, at best, a slight activator of FucT at concentrations around 100 μM. Higher concentrations of Mn^{2+} began to exert deleterious effects on enzyme activity. While it is not possible to be certain without a X-ray crystal structure of α1,3/4 FucT, these data suggest *H. pylori* α1,3/4 FucT is a metal independent GT with a probable GT-B fold type structure. The GT-B fold enzymes are generally not reliant on a metal co-factor, however many demonstrate higher levels of activity in the presence of a metal activator (Hu and Walker, 2002). In addition, when metal does promote activity, it may not bind by coordination to a DXD motif in GT-B fold enzymes (Qasba *et al.*, 2005).

3.1.6 Mass Spectrometry Analysis

Based upon the results observed on the effect of a panel of metal ions on α1,3/4 FucT activity, the possible association of Mg^{2+}, Mn^{2+}, and Ca^{2+} with α1,3/4 FucT was assessed using ICP-MS. ICP-MS is a highly sensitive technique capable of detecting trace levels of certain elements by using high temperature plasma to discharge positively charged ions (Thomas, 2001). The protein sample is injected in aqueous solution, and



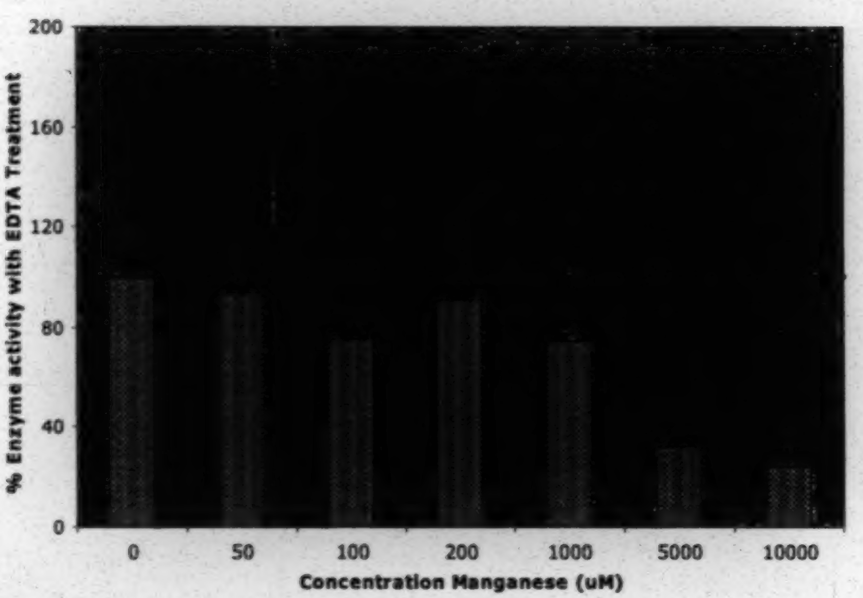


Figure 3.8 The effect of various concentrations of magnesium and manganese on EDTA treated purified UA948$^{1-428}$ α1,3 FucT. UA948$^{1-428}$ α1,3/4 FucT was treated with 2.5 mM EDTA for 12 hrs at 4°C to chelate exogenous metals from the reaction mixture. Metal was then added at the concentration indicated and the reaction mixture was allowed to sit for 3 hrs at 4°C. α1,3 fucosyltransferase activity is shown as a percentage of the UA948$^{1-428}$ FucT treated with 2.5 mM EDTA. Blue line represents activity of EDTA treated FucT. Results shown are the average of a minimum of three trials at each metal ion concentration with the standard deviation as indicated.

then aerosolized. The sample is excited by high energy plasma, and ionized. The presence of the divalent cations such as Mg^{2+} are detected by a quadrapole mass analyzer (Thomas, 2001). This allows for verification of their presence and for quantification. Analysis of the purified FucT enzyme showed little evidence of metal association (Ca^{2+}, Mg^{2+}, Mn^{2+}) with the protein (Table 3.2). The results showed none of the divalent cations detected to be present at a concentration equivalent to the calculated protein concentration. Of the three cations, Mg^{2+} was detected at the highest level, calculated to equal one Mg^{2+} per 10 FucT molecules. This low level of metal ion association with FucT provides additional evidence that no metal is required for *H. pylori* α1,3/4 fucosyltransferase activity. To ensure that the metal was not dissociating from α1,3/4 FucT during the dialysis step, the ICP-MS analysis was repeated using protein samples, which were not subjected to the overnight dialysis, but instead to buffer exchange using Amicon filters (10000 MWCO). The pattern of results mirrored those of the samples without dialysis treatment, with detection of very low levels of metal ions (Mg^{2+}, Ca^{2+} or Mn^{2+}). Taken together, these data document the first instance of metal independence in a bacterial fucosyltransferase.

3.1.7 pH Dependence of FucT

While the α1,3 and α 1,3/4 FucTs have been extensively characterized by our laboratory, the pH stability is one aspect of these enzymes that has not been previously evaluated. Given that the stability of the FucTs may be relevant to further biochemical characterization, the activity of the α1,3/4 FucT enzyme at a series of acidic pH's was assessed (Fig 3.9). The α1,3/4 FucT proved to be remarkably tolerant of acidic pH.

Table 3.2 Molar ratio of metal ions to protein molecules based on ICP-MS analysis of FutA from strains UA948 and 11639 for metal content. Samples were run with addition of an excess of nitric acid (HNO_3) to facilitate release of metal ions. The molar ratios were calculated using the theoretical mass of the FucT protein and the molecular weight of the applicable metal ion.

FucT	**Buffer exchange**	**Calcium (Ca^{2+})**		**Magnesium (Mg^{2+})**		**Manganese (Mn^{2+})**	
		normal	HNO_3	normal	HNO_3	normal	HNO_3
11639	Dialyzed	0.0314	0.4335	0.1327	7.2323	0.0098	1.2013
11639	Amicon filter	0.0192	0.1600	0.0742	0.3475	0.0088	0.0598
UA948	Amicon filter	0.0049	-0.0275	0.0953	0.1310	0.0057	-0.0180

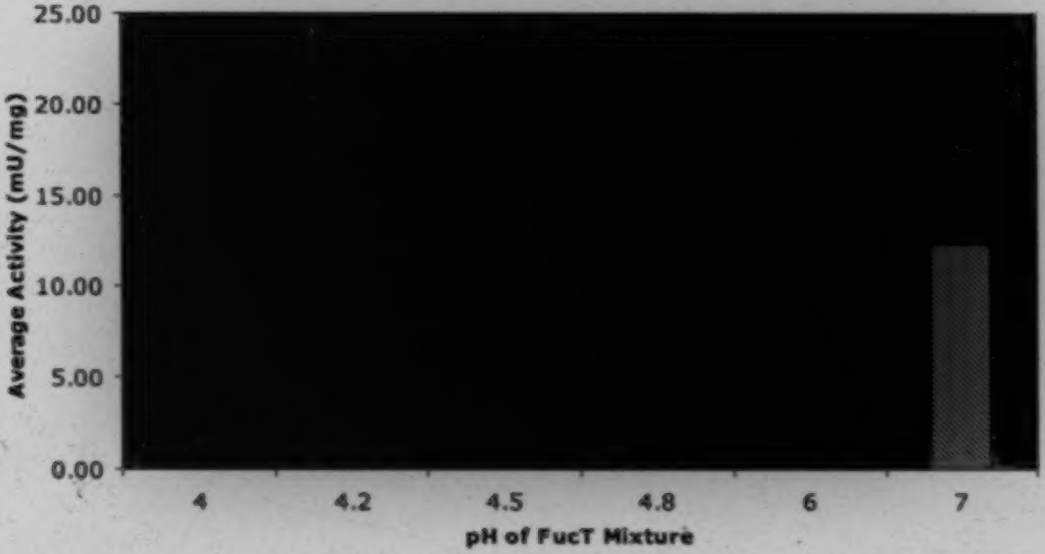


Figure 3.9 Effect of acidic pH on activity of purified UA948$^{1-428}$ α1,3 FucT. Purified UA948$^{1-428}$ α1,3 FucT was incubated in reaction mixture at the indicated pH for 3 hrs at 4°C followed by analysis of fucosyltransferase activity. Each value represents enzyme specific activity (mU mg^{-1}) as an average of three determinations with the standard deviations indicated. Assays at pH 4 to 6 were performed in 100 mM citrate buffer, as represented by the grey bars; assays at pH 7 were performed in 100 mM HEPES buffer, as represented by the crosshatched bar.

Relatively high levels of activity, as compared to standard assay condition (pH 7.0) are retained across the range of pH 4.5 to 9. Values for pH 1 → 6 are shown in Fig. 3.9. Activity at pH 4.5 was 51.3 % ± 6.3 % of UA948 FucT activity in standard assay conditions. α1,3/4 FucT is very stable in basic solutions, as it retained 100 % activity at pH 9.0 compared to standard assay conditions. The tolerance of this FucT to pH changes may reflect the *H. pylori* habitat in the human GI tract. However, as α1,3/4 FucT is not a surface exposed protein, an alternate explanation for its wide range of pH tolerance is perhaps more probable. The enzyme is most likely anchored to the inner membrane (Ma *et al.*, 2003), so it is not expected that FucT would be required to tolerate the drastic changes of pH encountered by *H. pylori* in its passage through the acidic stomach to the more neutral mucosal layer. It may be that acid tolerance of the FucT is simply a general characteristic of the type of enzyme, rather than a reflection of any environmental challenges faced by the FucT. The human FucT V has also shown stability at acidic pH levels (Murray *et al.*, 1996).

3.2 *Immunofluorescence Microscopy*

Immunofluorescence microscopy was performed to demonstrate the localization of the α1,3 and α1,3/4 FucT protein in the bacterial cell. The cellular localization of human GTs has been previously studied using immunofluorescent microscopy (Berger *et al.*, 1993), however, to my knowledge the technique has not been previously used for a bacterial GT. Both α1,3 and α1,3/4 FucT enzymes tagged with the C-terminal his tag were expressed in *E.coli*, as expression levels of FucT in *H. pylori* were expected to be insufficient for immunodetection. Additionally, immunodetection in *H. pylori* would have required adding a his tag to the WT α1,3/4 FucT. As a Gram negative bacterium, found naturally in the human gastrointestinal tract, *E. coli* was thought to be an appropriate model organism to investigate the localization of α1,3/4 FucT. Fluorescent FucT was easily observed, using the methanol fixation method (Hiraga *et al.* 1998) and probing for the α1,3/4 FucTs his-tag with fluorescent labeled antibodies. The images show that FucT appears to localize to the membrane of the bacterium (Fig. 3.10). This agrees with previous localization studies performed in our laboratory (Ma *et al.* 2006). The images show a high expression level, and the pattern of fluorescence distribution along the membrane would indicate that there is not a single site of FucT localization. There are, however, typically one to three focal points of fluorescence in many bacteria. This may indicate the proteins associated with the synthesis of the LPS occupy discrete locations at the cell surface. An alternative possibility is that the overexpression of protein may result in disruption of normal protein trafficking in the cell, leading to the presence of FucT inclusion bodies. The successful fluorescence visualization of FucT achieved through this experiment in an *E. coli* system suggests the possibility of attempting similar studies using an *H. pylori* expression system.

Insert Figure 3.10

3.3 *Transcriptional Analysis of fut Genes*

3.3.1 Growth of *H. pylori* strains

H. pylori strains with different Le antigen phenotypes were used in this study, to determine if there was any relationship between transcription patterns and the Lewis phenotype expressed on the cell surface. Several non-typable Le antigen strains were included in the study (strains 2511609, 12C2, PU32). In addition, several Le antigen expressing strains were also included: strains 26695 (Le^{XY}), 11639 (Le^{X}), G27 (Le^{Y}) and CCUG (Le^{Y}). Strains not expressing Le antigens are rare among clinical isolates, accounting for ~5% of strains from symptomatic patients (Rasko *et al.*, 2001). The non-typable strains often did not grow as well in laboratory culture as the Le antigen expressing strains. This may be due, in part, to the increased number of passages of the Le antigen expressing strains leading to adaptations to suit laboratory culture conditions. By determining the transcription patterns of the *fut* genes from non-typable strains, it may be possible to determine the cause of variations in Le antigen expression of these strains. Two main possibilities existed: that the *fut* genes required for Le antigen synthesis were not transcribed; or that post-translational processing and/or variations in the amino acid sequence resulted in a functionally inactive protein.

3.3.2 *fut* Gene Characteristics

A survey of the TIGR Comprehensive Microbial Database (www.tigr.org) for annotated *H. pylori* genomes (strains 26695 and J99) revealed that the LPS synthesis genes are not clustered. The three fucosyltransferase genes are found in different locations on the chromosome, as are the putative genes in the biosynthetic pathway of Le Ag synthesis. For example, the GDP-D-mannose dehydratase (HP0044) gene, an enzyme thought to be crucial in the synthesis of GDP-fucose, is far removed from *futA/B/C*, and from other LPS synthesis genes. As described previously, this scattered distribution of the *fut* genes throughout the genome is unusual for O-antigen expressing bacteria (see Samuel and Reeves, 2003). *E. coli*, *S. enterica*, and *P. aeruginosa* are among the species that encode O-antigen genes in clustered groupings. The *fut* genes are found at the following locations on the 26695 genome: *futA* at ORF HP0651, *futB* at ORF HP0379, and *futC* at ORF HP0093/94. The distribution of the three fucosyltransferase genes is similar in the J99 genome sequence. The reason for the separation of the fucosyltransferase genes is unknown.

3.3.3 Transcriptional Patterns of *fut* Genes

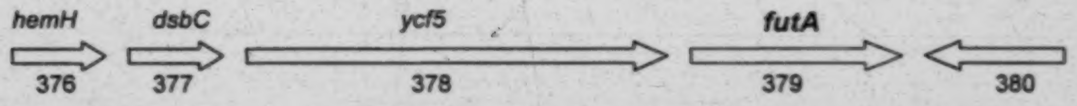
The transcription of *futA*, *futB* and *futC* genes was investigated by RT-PCR to determine whether these genes were co-transcribed with upstream genes. To analyze the transcription patterns, primer pairs for adjacent coding regions were designed for use in cDNA amplification. The primers were created to amplify short regions of cDNA which incorporated a short region of a downstream gene, a short region of the possible co-transcribed upstream gene, and any non-coding bases between the two coding regions. The ORF immediately downstream of all three *fut* genes is encoded on the opposite strand of DNA, and as such these ORFs were not assessed for co-transcription with the *fut* genes. It is interesting to note the proximity of the *fut* genes to putative ORF's involved with iron metabolism.

3.3.3.1 *futA* Analysis

The *futA* gene was shown to be co-transcribed with the upstream genes *yct5*, *dsbC* and *hemH* in several strains (Figs. 3.11 & 3.12). This expression may be related to the Le antigen expression pattern of the bacterial strain in question. Strain 26695, which expresses both Le X and Le Y, shows consistent evidence of transcribing *futA* with all three upstream genes. The non-typable *H. pylori* strains and strain G27 show weaker evidence of *futA* transcription, which might be expected given their lack of expression of Le antigens. There is still indication of co-transcription with the *yct5* and *dsbC* genes, immediately upstream, however there is no PCR product of *dsbC* with *hemH*. This suggests that *futA* is potentially still transcribed in these strains, albeit with a different pattern of transcription. As such, it is possible that slip-strand synthesis or post translational modifications of the FucT enzyme are responsible for the lack of expression of typable Le antigens in these strains.

3.3.3.2 *futB* Analysis

The transcription of *futB* appears to be co-transcribed with the *serB* gene immediately upstream (Figs. 3.13 & 3.14). No strains showed evidence of co-transcription with *pfr*. Similar to the *futA* gene, there is no evidence of transcription in non-typable strains of *H. pylori*, perhaps due to slip-strand insertion of a nucleotide into the upstream sequence. It has been well documented that slip strand synthesis can interrupt *fut* gene translation, and lead to high ON/OFF variation in Le antigen expression observed even within the population of one strain of bacterium (Appelmelk *et al.*, 1999). However, there has not been as much study of possible mechanisms for altering transcription of the genes involved in Le antigen synthesis. *SerB* is co-transcribed with



		Operon Region			
Strains	**Le Antigens**	**Upstream – *hemH***	***hemH* – *dsbC***	***dsbC* – *ycf5***	***ycf5* – *futA***
26695	X Y	-	✓	✓	✓
11639	X	-	?	?	✓
G27	Y	-	-	✓	✓
CCUG	Y	-	?	✓	✓
25191106	non-typable	-	-	✓	✓
PU32	non-typable	-	-	✓	✓

Figure 3.11 Summary of RT-PCR from *futA* operon in various strains of *H. pylori*. PCR primer pairs were designed to amplify the DNA / cDNA between two adjacent ORFs. The pairs are listed under the Operon Region with the following symbols to represent the presence or absence of PCR products of the correct size: "-" = no amplification from cDNA; "?" = variable amplification from cDNA; "✓" = consistent amplification from cDNA. The *H. pylori* strains and their Le antigen status are indicated in the left-hand column. ORF 380 is encoded on the opposite strand of DNA, and so was not included in RT-PCR analysis.

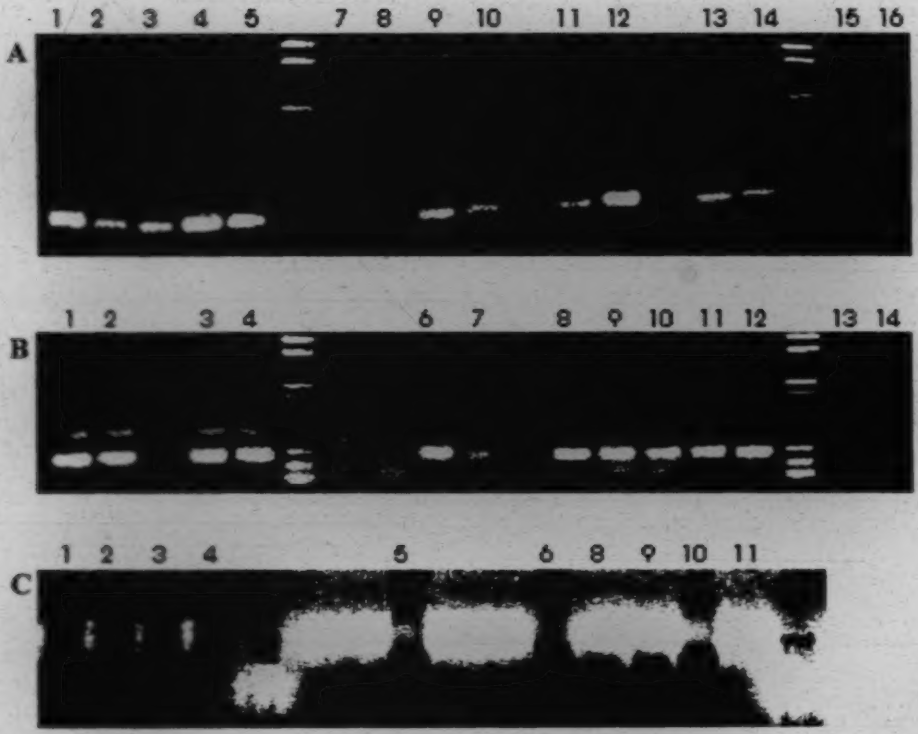
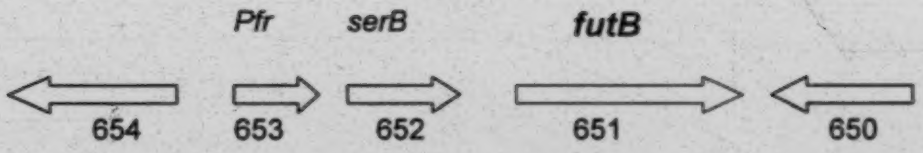


Fig. 3.12 Images of *futA* operon RT-PCR product following electrophoresis separation. PCR amplified product was separated by gel electrophoresis at 110 V for 1 hr and visualized by EtBr staining. The gels show representative amplification product from RT-PCR of cDNA, with DNA positive controls and –RT cDNA negative controls. **A. *futA* – *ycf5*:** Lane 1-5: DNA control; Lane 7-14: cDNA amplification: Lane 7, 12 – 26695 cDNA, Lane 8 - 11639 cDNA (old preparation), Lane 9, 13 – G27 cDNA, Lane 10, 14 – 25191106 cDNA, Lane 11 – PU32 cDNA; Lane 15-16: no RT negative control. **B. *dsbC* – *ycf5*:** Lane 1-4: DNA control; Lane 6-12: cDNA amplification: Lane 6,11 – G27 cDNA, Lane 7-12 - 25191106 cDNA, Lane 8 – pU32 cDNA, Lane 9 – 26695 cDNA, Lane 10 - 11639 cDNA; Lane 11 – G27; Lane 13-14: no RT negative control. **C. *hemH* – *dsbC*:** Lane 1-4: DNA control; Lane 5-11: cDNA amplification: Lane 5 – G27 cDNA, Lane 6 - pU32 cDNA, Lane 8 – 26695 cDNA, Lane 9 – 11639 cDNA, Lane 10 – G27; Lane 11 – 25191106 cDNA



		Operon Region		
Strains	**Le Antigens**	**Upstream – *pfr***	***pfr* – *serB***	***serB* – *futB***
26695	X Y	-	-	✓
11639	X	-	-	✓
12C2	non-typable	-	-	?
25191106	non-typable	-	-	-
PU32	non-typable	-	-	-

Figure 3.13 Summary of RT-PCR results from *futB* operon of various strains of *H. pylori*.
PCR primer pairs were designed to amplify the DNA / cDNA between two adjacent ORFs. The pairs are listed under the Operon Region with the following symbols to represent the presence or absence of PCR products of the correct size: "-" = no amplification from cDNA; "?" = variable amplification from cDNA; "✓" = consistent amplification from cDNA. The *H. pylori* strains and their Le antigen status are indicated in the left-hand column. ORF 650 is encoded on the opposite strand of DNA, and so was not included in RT-PCR analysis.

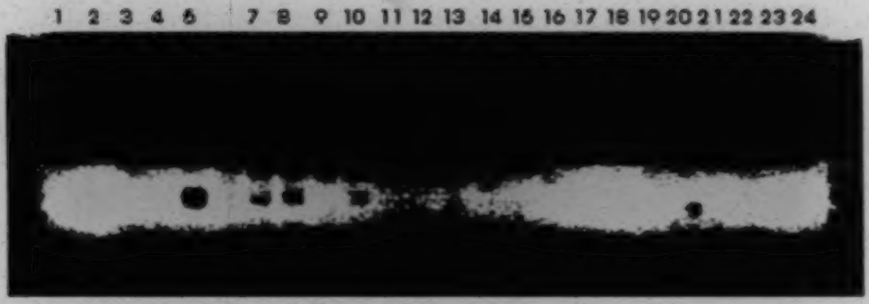


Fig. 3.14. Images *futB* operon RT-PCR product following electrophoresis separation. PCR amplified product was separated by gel electrophoresis at 110 V for 1 hr and visualized by EtBr staining. The gels show representative amplification product from RT-PCR of cDNA, with DNA positive controls and –RT cDNA negative controls. Lane 1-4: UreA positive control; Lane 1 – 26695 DNA, Lane 2 – 26695 cDNA, Lane 3 – UA948 cDNA, Lane 4 – no RT negative control; Lanes 5 – 10: *serB-futB*; Lane 5 – 26696 DNA, Lane 6 – 26695 cDNA (old preparation), Lane 7 – UA948 cDNA, Lane 8 – 12C2 cDNA, Lane 9 – PU32 cDNA, Lane 10 - no RT negative control; Lanes 11 – 17: *pfr-serB*; Lane 11-12 – 26696 DNA, Lane 13 – 26695 cDNA (old preparation), Lane 14 – UA948 cDNA, Lane 15 – 12C2 cDNA, Lane 16 – PU32 cDNA, Lane 17 - no RT negative control. ; Lanes 11 – 17: Upstresm - *pfr*; Lane 18 – 26696 DNA, Lane 19 – 26695 cDNA (old preparation), Lane 20 – UA948 cDNA, Lane 21 – 12C2 cDNA, Lane 22 – PU32 cDNA, Lane 24 - no RT negative control.

futB in strains of *H. pylori* with different Le antigen expression patterns. One non-typable strain did show evidence of transcription, indicating that *futB* may undergo slip-strand synthesis during translation in at least some of the non-typable strains. It is likely that there may be multiple levels of control of FucT expression, and that interruption at any one of these may lead to the loss of Le antigen expression.

3.3.3.3 *futC* Analysis

The annotated sequences of both strains 26695 and J99 show no evidence of upstream genes on the same coding strand of the chromosome as *futC*. Initial RT-PCR analysis showed no evidence of co-transcription with the region upstream (ORF 0095). Taken together, these data indicate that *futC* is most likely to be independently transcribed in *H. pylori*.

4 Discussion

4.1 *Summary of Metal Dependence*

There have been no reports in the scientific literature regarding the metal dependence of any prokaryotic fucosyltransferase. Unfortunately, although X-ray crystal structures have been resolved for several other microbial GTs, no common theme of fold-type or metal dependence has been determined from these structures (Breton *et al.*, 2006). The studies which comprise this thesis show *H. pylori* α1,3/4 FucT to be a metal independent GT. This contributes to the understanding of the catalytic mechanism of this enzyme and suggests the enzyme structure most likely resembles that of other metal independent GTs, such as those belonging to the GT-B fold family (Hu and Walker, 2002).

The *H. pylori* α1,3/4 FucT is the first bacterial fucosyltransferase to be studied in detail. Mutational analysis and domain swapping studies have significantly increased understanding of the Type I acceptor specificity recognition site of *H. pylori* α1,3/4 FucT (Ma *et al.*, 2003; Ma *et al.*, 2005), however, less is understood about the nucleotide-sugar binding site. The mutation and metal dependence studies undertaken in this thesis have furthered understanding of the donor requirements. *H. pylori* α1,3/4 FucT contains EXD motifs, which are analogous to the DXD motif found in other GTs, and frequently provide a coordination site for Mn^{+2} co-factor (Unligil and Rini, 2000).

Our initial mutational analysis of the EXD motifs showed that, while necessary for WT enzymatic catalysis, no single residue was imperative for fucose transfer. The mutational analysis of the EXD motifs in the α1,3/4 FucT peptide sequence indicates that while the acidic residues contribute to WT activity levels, their deletion does not completely abolish activity, as would be expected with a typical DXD motif (Hu and Walker, 2002). In addition, of the three potential candidates for the DXD motif, no particular motif was shown to be significantly more deleterious to activity. This suggests that the α1,3/4 FucT in *H. pylori* is not representative of a typical GT-A family enzyme and that it may not absolutely require a metal ion for activity. These suggestions were further confirmed by the metal dependence assays.

The metal dependence assays demonstrated that the only metal to have an activating effect on FucT was Mg^{2+} and all other divalent ions assessed were found to have negative impact on FucT activity. This was surprising, as a large number of GTs are either activated or have an absolute requirement for a Mn^{2+} ion, which can typically be substituted for with Mg^{2+} (Charnock and Davies, 1999; Tarbouriech *et al.,* 2001). The work in this thesis has shown that *H. pylori* FucT differs from human FucT III and FucT V in its metal ion requirements. Both FucT III and FucT V catalyze the final step in the synthesis of Le antigens (Le^{a} and Le^{X}, respectively), and have been demonstrated to be strongly activated by Mn^{2+} (Murray *et al.,* 1996; Palma *et al.*, 2004). *H. pylori* FucT showed no analogous activation by Mn^{2+} and was in fact deleteriously affected by its addition. Mn^{2+} and Mg^{2+} have been proposed to be able to commonly substitute for one another as they can both assume octahedral geometry, and are of similar size (Murray *et al.*, 1996). Why these two similar cations would have opposite effects on the enzyme activity is not well understood, however, it has been noted with several other GTs that different divalent cations may have different effects on enzyme activity (Palma *et al.*, 2004; Schwyzer and Hill, 1977; Taniguchi and Makita, 1984). One possible explanation is that the effect of the divalent cation may be related to the strength of the metal ion – GDP-fucose bond. The stability of the ADP-metal ion bond increases in the following order: $Ca^{2+} < Mg^{2+} < Co^{2+} < Mn^{2+} < Zn^{2+} < Ni^{2+} < Cu^{2+}$ (Taqui and Martell, 1967), and it would be expected to follow the same trend with GDP (Murray *et al.* 1996). Interestingly, the three metal ions with a stronger bond than Mn^{2+}, Zn^{2+}, Ni^{2+}, and Cu^{2+}, are the three that have been well documented to have a deleterious effect on the transferase activity in other GTs (Murray *et al.*, 1996; Palma *et al.*, 2004; Schwyzer and Hill, 1977; Taniguchi and Makita, 1984). Of the three, it is the ion forming the strongest bond, Cu^{2+}, which is found to have the most deleterious effect (Palma *et al.*, 2004; Schwyzer and Hill, 1977; Taniguchi and Makita, 1984). It can therefore be proposed that the *H. pylori* α1,3/4 FucT enzyme's Mg^{2+} activation favours a weaker metal-GDP bond than the Mn^{2+} ion, thereby offering an explanation as to why the Mn^{2+} might be deleterious to this FucT.

ICP-MS analysis was consistent with the lack of complete dependence of *H. pylori* α1,3/4 FucT on a metal ion. If a divalent ion was bound to the α1,3/4 FucT, it would be expected to show a one to one ratio with the enzyme in this mass spectrometric analysis, which is specialized for the detection of metal ions. The mass spectrometric analysis for Ca^{2+}, Mn^{2+}, and Mg^{2+} showed that Mg^{2+} was present in the samples at a considerably higher concentration than the other metal ions. The ratio of Mg^{2+} to α1,3/4 FucT enzyme was still low, at a ratio of 1:10 metal to protein. Thus, it is not possible to conclude if the metal ion is only weakly bound to the enzyme, or if no Mg^{2+} is present.

The data in this thesis suggest that the *H. pylori* α1,3/4 FucT most likely has a fold structure similar to the GT-B fold family of enzymes. An alternative explanation would be that the *H. pylori* α1,3/4 FucT falls within the less well characterized GT-C or GT-D fold family of enzymes. Further elucidation of the *H. pylori* α1,3/4 FucT structure by X-ray crystallography would aid greatly in identifying how the enzyme is able to catalyze the transfer of the fucose molecule, while offsetting the negative charge imparted upon the nucleotide leaving group. As *H. pylori* α1,3/4 FucT is the first fucosyltransferase from a bacterial system that has been characterized for metal dependence, the results could be interpreted to mean that metal independent FucT's are more common in the prokaryotic kingdom than in the eukaryotic kingdom. Still, more bacterial FucT enzymes need to be studied before any such conclusion can be definitively drawn. Of the mammalian fucosyltransferases that have been well characterized to date, human FucT III and V, have both been shown to require Mn^{2+} for full activity (Murray *et al.*, 1996; Palma *et al.* 2004). A survey of GTs with resolved crystal structures revealed there to be more diversity in the GT-fold in prokaryotes than in eukaryotes. GT crystal structures from prokaryotes belong to both the GT-A and GT-B family, and show DXD mediated metal dependency in only some of the GT-A like structures (Breton *et al.,* 2006; Chiu *et al.*, 2004). Furthermore, bacterial GTs have no apparent preference for inverting- versus retaining-type catalysis (Breton *et al.* 2006). In contrast, eukaryotic GT crystal structures unilaterally belong to the GT-A fold family, and all show metal dependence (Breton *et al.* 2006). The findings in this thesis also lend support to the classification of the *H. pylori* FucT in the GT family 10, as this family is predicted to have the GT-B super-family structure.

4.2 *pH Dependence of FucT*

The high level of acid tolerance of the α1,3/4 FucT observed in these studies is of particular interest given that this bacterium inhabits the acidic environment of the human stomach. While a direct relationship between the acid tolerance of the α1,3/4 FucT and *H. pylori* being found in the stomach seems doubtful, it will be interesting to learn whether this acid tolerance is widespread in bacterial GTs involved with LPS synthesis. The stability of FucT at lower pH levels would also be a further indication of its metal independent activity, because acidic pH increases the likelihood of dissociation of metal ions from protein complexes (Palma *et al.*, 2004). However, Murray *et al.* (1996) found human FucT V to be stable from pH 3.5 to 10, a similar range to the *H. pylori* α1,3/4 FucT enzyme. Since human FucT V is a metal dependent GT, this would indicate that a α1,3/4 FucT may show a wide range of pH stability, regardless of its particular metal ion requirements. In the human stomach, the majority of *H. pylori* are thought to inhabit the mucosal layer covering the gastric epithelium, where a relatively neutral pH is maintained (Bahari *et al.*, 1982; Schreiber *et al.*, 2004). In the Mongolian gerbil model of *H. pylori* infection, only a very small proportion of bacteria were exposed to, and survive, the acidic pH of the stomach (Schreiber *et al.*, 2004). Given the environmental niche inhabited by *H. pylori,* an alternative explanation for the acid tolerance of the *H. pylori* α1,3/4 FucT enzyme is that while the intracellular pH of the bacterium is assumed to be relatively constant, acid tolerance of the FucT enzyme may assist the bacteria in tolerating any localized pH fluctuations that *H. pylori* might encounter during passage from the low pH environment of the stomach to the more neutral environment found in the epithelial and mucosal layers

4.3 *Purification of FucT*

The purification of full length α1,3 FucT enzyme carrying a His-tag was successful in achieving significantly purified protein, which retained good levels of activity. The level of purity was highest using a low pressure, manual system for purification. The purification of full length α1,3 FucT was complicated by its very rapid elution of the protein from the column resins. The elution of α1,3 FucT from the Ni^{2+} column occurred at a very low concentration of imidazole and is evidence of a low binding affinity of the protein to the column resin, despite its C-terminal histidine tag. This may be due to either its low solubility in aqueous solution, or a partially obstructed His-tag. It is possible that tertiary protein folding or the hypothetical dimerization at the C-termini of FucT, which has been suggested previously (Ge *et al.*, 1997; Ma *et al.*, 2006; Martin *et al.*, 1997), could obstruct the His-tag, and may thus interfere with binding to the Ni^{2+} chelating column resin. To increase the binding affinity of the protein to the column resin, two possible approaches exist. The first relies on modifying the protein, as the histidine tag could be lengthened to 10 residues to reduce the probability of it being folded within the tertiary structure of the protein. A second method would be to use an alternate column resin. Cobalt (Co^{2+}) and other transition metal resins have higher affinity for histidine residues and can assist purification of proteins with low binding affinities to Ni^{2+} resin (Yip and Hutchens, 1996). The most significant drawback of the purification method employed in this thesis was the tendency of the protein to break down on the column when reconditioned resin was used. The α1,3/4 FucT enzyme constructs $UA948^{1\text{-}428}$ and $11639^{1\text{-}441}$ of Ma *et al.* (2006) offered increased stability and purity, and as such are currently thought to be better candidates for crystallization studies, compared with the purified full length α1,3 FucT enzyme. Previous studies of different GTs have also encountered difficulty in obtaining high levels of expression and subsequent purification (reviewed in Breton *et al.,* 2006). Although the current progress with FucT enzyme purification may seem to be of limited success, it must be considered in light of the challenges which have been met with purification of other GTs.

4.4 *Immunofluorescence Microscopy*

The fluorescence images obtained show a very high level of FucT expression with small focal points of fluorescence throughout the cell. It is possible that these points represent discrete clusters of FucT protein, however a second explanation is that these structures represent inclusion bodies. Nonetheless, this represents the first visualization of α1,3/4 FucT expression within a bacterial system. One interesting feature that this technique demonstrates is the very high level of enzyme expression that has occurred and is still present with in the bacterial cells, particularly if the fluorescent focal points represent inclusion bodies. Earlier assessment of α1,3/4 FucT expression relied on the more quantifiable immunoblotting and SDS page stained with Coomassie blue. These experiments, however, did not seem to indicate such a high level of FucT expression. While the negative control cells showed the fluorescently labeled antibody was very specific for the mouse α-pentahistidine antibody, the level of fluorescence obtained indicates that a considerable amount of the secondary antibody is capable of binding to the primary antibody, giving rise to a very high level of fluorescence.

4.5 *Transcription Patterns of H. pylori fut Genes*

The patterns of transcription of the three *H. pylori fut* genes were investigated by RT-PCR. Analysis of the ORFs surrounding the *futC* gene, which encodes the α1,2 FucT, indicated that there was unlikely to be any upstream genes co-transcribed with *futC*. This observation was confirmed by the fact that RT-PCR demonstrated no evidence of co-transcription with the upstream region. The *futA* and *futB* genes encoding the α1,3/4 FucT enzymes in *H. pylori* were transcribed with upstream genes. *FutA* is apparently co-transcribed with all three ORF's, immediately upstream and in the same orientation. These genes are not related to LPS synthesis, but rather to cytochrome C biogenesis (*yct5*), thiolidosulphate interchange (*dsbC*) and iron metabolism (*hemH*). The *futB* gene is co-transcribed with the *serB* ORF, a gene for phosphoserine phosphatase. As has been noted, *H. pylori* is unusual in its lack of clustering of O-antigen related genes. This thesis shed no light on reasons for the separation of the *futA, futB* and *futC* genes from each other. There is no pattern in the types of putative genes encoded by the ORF's surrounding the individual *fut* genes. *H. pylori* strains not expressing typable Le antigens showed weaker evidence of transcription. These results indicate that lack of FutA and FutB activity in the non-typable strains is due, at least in part, to lack of RNA transcription as found in those *H. pylori* isolates expressing distinct Le antigen types. As frameshift mutations are common in *H. pylori* (Ge *et al.* 1999; Appelmelk *et al.* 1999), this may explain the lack of transcription and subsequent translation that is observed in the non-typable strains.

4.6 *Future Directions*

While the work in this thesis suggests that activity of α1,3/4 FucT is metal independent, there are still a number of unanswered questions regarding the enzyme's structure and function. To date, obtaining α1,3/4 FucT crystals suitable for X-ray diffraction has not been successful. This adds to the importance of pursuing alternative studies to elucidate the α1,3/4 FucT structure. Nonetheless, the resolution of a α1,3/4 FucT crystal structure is required for a full understanding of tertiary structure of the enzyme and for concrete evidence as to which GT fold family this bacterial α1,3/4 FucT belongs. The observation that Mg^{2+} significantly increases the non-metal treated FucT activity indicates there is interaction between Mg^{2+} and the enzyme. This interaction could aid in stabilizing the protein during crystallization attempts. A crystal structure would help to elucidate whether the Mg^{2+}-FucT interaction is of a specific or non-specific nature.

Although Palma *et al.* (2004) did not look at metal:Fut III interactions directly by mass spectrometry, they did find that the Mn^{2+} binds to GDP-fucose in a 1:1 ratio. An interesting follow-up to this study would be to assess whether the Mg^{2+} binds to the GDP-fucose in a similar manner, as this would be relevant to the Mg^{2+} activation which was observed with *H. pylori* FucT.

The localized focal points of fluorescence observed in the immunofluorescent microscopy images may represent the presence of inclusion bodies. This possibility could be examined further by lysing the cells, followed by centrifugation and checking the liquid fraction for fluorescence. This microscopy study has illustrated the feasibility of using the histidine-tagged FucT in further immunofluoresence studies in an *H. pylori* expression system. In addition, the high affinity of the labeled secondary antibody will facilitate detection of the FucT enzyme in an expression system more representative of the WT expression levels.

The final area of investigation in this thesis was the transcriptional analysis of the three *H. pylori fut* genes. To further this work, promoter analysis of the proposed co-transcribed ORF's should be performed. In addition, transcription of a number of *H. pylori* genes has been shown to be influenced by acidic pH (Merrel *et al.* 2003). While Merrel used spotted DNA-microarrays to assess transcription of the *H. pylori* genome, it would be of interest to perform a more detailed study on the LPS associated genes. One such set of experiments would be of interest to closely investigate the effect of pH on *fut* transcription and correlate this to the Le antigen expression in acidic and neutral conditions.

Although *H. pylori* was only cultured less than 25 years ago, a considerable amount of information about many aspects of the bacterium and its interaction with the human host has been published. Nevertheless more remains to be done, particularly concerning the role that Lewis antigens play in the infectious process and why the bacterium produces molecules with such obvious similarities to those found in the human stomach.

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